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EVALUATION OF OSTEOPRODUCTIVE BIOMATERIALS: ALLOGRAFT, BONE INDUCING AGENT, BIOACTIVE GLASS, AND CERAMICS

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Academic Dissertation

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YOU

By Edgar A. Guest

You are the fellow that has to decide
Whether you'll do it or toss it aside.

You are the fellow who makes up your mind
Whether you'll lead or linger behind;

Whether you'll try for the goal that's far,
Or just be contented to stay where you are.

Take or leave it, here's something to do!
Just think it over—it's all up to you.

What do you wish? To be known as a shirk,
Or known as a good man who's willing to work?

Scorned as a loafer, or praised by your chief,
Rich man or poor man or beggar or thief?

Eager and earnest or dull through the day?
Honest or crooked? It's you who must say.

You must decide in the face of the test
Whether you'll shirk or live it your best.

CONTENTS

1. ABSTRACT

This thesis focuses on clinically relevant evaluations of natural and synthetic grafting materials. The intent was to develop a sound methodology to evaluate biomaterials considered for clinical use and to provide an insight on the different grafting stategies to enhance bone formation.
The most traditional technique to bridge large defects involves the use of segments of frozen allogenic cortical bone. In this instance, it is crucial that cortical allografts retain biomechanical properties, allowing the repaired bone to withstand forces generated during weight bearing. In the first part of this study, the influence of long-term storage of allografts frozen in saline or dry containers was evaluated in bending and torsion. Within pairs of canine metacarpal bones, metatarsal bones, and ribs, one bone was frozen in a dry plastic container, while the other was immersed in a normal solution of sodium chloride for one year. The energy absorbed at failure and the ultimate displacement of all bones tested in bending were increased by 25%, to 30% and 18%, to 24% respectively, when the bones were frozen in isotonic saline solution. Corticocancellous grafts frozen in normal saline solution are biomechanically less fragile and brittle than grafts stored in plastic without saline solution.
Where biomechanical strength is not crucial, fresh autogenous cancellous graft is still considered the ”golden standard” when evaluating other biomaterials. However, the morbidity and additional surgical time associated with its collection, as well as the limited supply in each patient, have stimulated the search for substitutes. These have been classified as osteoinductive or osteoconductive, depending on their properties.
The second part of the study dealt with an osteoinductive graft substitute extracted from a human osteosarcoma cell line. The osteoinductive properties of this bone-inducing agent (BIA) were tested in the Latissimus Dorsi of five dogs. Because of its clinical relevance, we also evaluated this material after orthotopic implantation, in unicortical femoral defects created in four dogs. The effects of BIA were compared with bovine collagen I (the carrier), a gelatin capsule (negative control), and fresh autogenous cancellous graft (positive control), after orthotopic and heterotopic implantation. Bone formation was evaluated via serial radiographs, dual energy X-ray absorptiometry, histology, and histomorphometry. Ten milligrams of BIA did not induce bone formation six weeks after heterotopic implantation and failed to promote bone healing eight weeks after orthotopic implantation in dogs. The discrepancy between these results and those previously obtained in rodents may be related to immunogenic factors or to the dose of BIA used in this study.
The third part of the study comprised osteoconductive biomaterials considered for potential use in impaction revision hip arthroplasty in man. The general purpose of this study was to evaluate the use of biomaterials to expand or replace allografts, with a specific interest in the revascularization and incorporation of impacted morselized grafts. In study III, a model was designed to simulate the environment encountered in revision hip arthroplasty and study the biological properties of several impacted aggregates of large particles in the same animal (higher vertebrate). The model was evaluated in Study III and applied to study biomaterials in Studies IV and V.
A miniature impactor was designed to produce pellets of aggregates at a compactive effort similar to that generated in clinical impaction grafting. Twenty-two sheep underwent implantation of pellets into six metaphyseal defects in both rear limbs (Study IV). Another eight sheep underwent surgical implantation of four pellets in metaphyseal defects (Study V). Defects were sealed with polymethylmethacrylate in both phases. Healing of the defects was evaluated at seven weeks (n=11) and 14 weeks (n=19) with computed tomography, histology, and

histomorphometry. Complications in the initial phase (sheep in Study IV) included four femoral fractures and migration of the cement seal (18/102 defects). No complications occurred in the later phase (sheep in Study V). Although no difference was found between left and right limbs, osteogenesis and incorporation of biomaterials varied between implantation sites. Treatment site allocation was randomized according to a Latin square design for comparison of grafting materials. This model allows evaluation of several impacted aggregates (including large particles) in the same animal. It is particularly suitable for analyzing the biological properties of grafting materials prior to evaluation under loading conditions.

The osteoproductive properties of a new silicate-free idealized morselized bioactive glass (Corglaes®), alone or combined with allograft were evaluated in Study IV. Particle size distribution of all aggregates (except the autograft) approached the ideal logarithmic grading line, and all implants were impacted at a standard compactive effort. Treatment groups consisted of (1) empty defect (negative control), (2) autograft (positive control), (3) allograft (clinical control), (4) allograft idealized with Corglaes®, (5) 50 /50 allograft / Corglaes®, and (6) Corglaes®. Healing of the defects was evaluated at seven weeks (n=6) and 14 weeks (n=16) with computed tomography, histology, and histomorphometry. Remnants of Corglaes® were found within one of the defects 7 weeks after implantation. Defects filled with mixtures containing 50% or 100% Corglaes® were less dense, and contained less bone and more fibrous tissue than defects with allograft, autograft, or allograft idealized with Corglaes®. Allograft idealized with Corglaes® may be considered for impaction grafting in revision hip arthroplasty, as well as local delivery of antibiotics. However, further studies and potential revision of the agent are required before mixtures containing concentrations of Corglaes® equal to or higher than 50% can be recommended.

In Study V, the model was slightly adjusted to investigate, the properties of idealized, impacted tricalcium phosphate-hydroxyapatite (TCP-HA) aggregates, varying in chemical composition (ratio of TCP to HA) and particle size distribution (idealization with 8 versus 3 particle size ranges). Treatment groups consisted of: (1) allograft (clinical control), (2) 50/50 allograft / 80%HA/20%TCP idealized with 8 particle size ranges, (3) 50/50 allograft / 80%TCP/20%HA idealized with 8 sizes and (4) 50/50 allograft / 80%HA/20%TCP idealized with only 3 particle sizes. At 14 weeks, the pixel value measured with computed tomography in all defects containing synthetic agents was higher than in defects filled with allograft alone ($p<0.01$). Defects containing the idealized mixture of 80%HA/20%TCP granules (group 2) achieved lower histological scores and contained less bone than the clinical control ($p<0.05$), whereas groups 3 and 4 did not differ from the control. Although all synthetic agents were osteoconductive, our results suggest that increasing the ratio of TCP over HA and limiting the number of particle size ranges to 3 instead of 8 improve the performance of impacted aggregates as graft expanders. Evaluation under loading conditions of morselized allograft expanded with 80%TCP/20%HA (BoneSave®) in 3 particle size ranges is warranted.

2. LIST OF ORIGINAL ARTICLES

This dissertation is based on the following publications which are referred to in the text by their Roman numerals.

1. **Study I:** Griffon DJ, Wallace LJ, Bechtold JE: Biomechanical properties of canine corticocancellous bone frozen in normal saline solution. Am J Vet Res 1995;56(6):822-825.

2. **Study II:** Griffon DJ, McLaughlin R, Hoskinson J: Effects of a Bone Inducing Agent derived from a cultured human osteosarcoma cell line after orthotopic and heterotopic implantation in the dog. VCOT 1996;9:22-28.

3. **Study III:** Griffon DJ, Dunlop DG, Howie CR, Pratt JNJ, Gilchrist TJ, Smith N: An ovine model to evaluate the biological properties of impacted morsellised bone graft substitutes. J Biomed Mater Res 2001;56(3):444-451.

4. **Study IV:** Griffon DJ, Dunlop DG, Howie CR, Gilchrist T, Salter DM, Healy DM: Early dissolution of a morsellised impacted silicate-free bioactive glass in metaphyseal defects. J Biomed Mater Res – Applied Biomater 2001;58(6):638-644.

5. **Study V:** Pratt JNJ, Griffon DJ, Dunlop DG, Smith N, Howie CR: Impaction grafting with idealised allograft and tricalcium phosphate – hydroxyapatite: Incorporation within ovine metaphyseal defects. Biomater 2002; 23: 3309-3317.

3. TERMINOLOGY, DEFINITIONS, AND ABBREVIATIONS

3.1. TERMINOLOGY AND DEFINITIONS

Allograft (adjective, *allogeneic*): living tissue transferred between two genetically different individuals of the same species (Stevenson 1999). Gorer was the first, in 1961, to suggest that *allograft* should replace the previously used term *homograft* (Gorer et al. 1961).

Alloimplant: nonviable tissue transferred between two individuals of the same species. Most allografts are actually alloimplants (Fitch et al. 1997).

Autograft (adjective, *autogenous* or *autogeneic*): a graft moved from one site to another within the same individual.

Bioactive: refers to a material that induces specific biological activity, as opposed to bioinert (Williams 1987).

Bone Morphogenetic Protein (BMP): generic name for osteoinductive proteins extractable from bone matrix with chaotropic agents, such as 4M-guanidine hydrochloride, and able to induce *de novo* bone formation (Urist 1990).

De novo *bone formation*: bone formation in a site devoid of osteoprogenitor cells.

Grade (of an aggregate): refers to the particle size distribution of the aggregate. A well-graded aggregate include particles of variable sizes, with a particle size distribution approaching the ideal curve or line (depending on the shape of particles) consistent with the particulate aggregate theory. Poorly graded aggregates are of more uniform size.

Heterotopic: refers to the placement of a graft in an anatomically inappropriate site (i.e. not in contact with bone). Also known as *ectopic* placement.

Idealization: correction of the particle size distribution of an aggregate to match the ideal logarithmic curve described by Fuller for spherical particles. According to the particulate aggregate theory, a modified curve should be used for irregular particles (Craig 1993).

Implant: nonviable material, such as bone, that has been frozen, freeze-dried, or sterilized by irradiation (Urist 1980, Burwell 1994).

Isograft (adjective, *isogeneic* or *syngeneic*): live tissue transferred from one twin into an identical (monozygotic) twin (Urist 1980, Stevenson 1999).

Orthotopic: Refers to anatomically appropriate (i.e. within bone) placement of a graft, as opposed to heterotopic.

Osteoconduction: process whereby microscopic and macroscopic scaffolding is provided for inward migration of cellular elements involved in bone formation (e.g. mesenchymal cells, osteoblasts, osteoclasts, and vasculature). Implanted bone acts as a structural framework ("trellis") for bone ingrowth (Fitch et al. 1997).

Osteogenesis: in a general sense, osteogenesis refers to bone formation with no indication of cellular origin: new bone may originate from live cells in the graft or cells of host origin (Stevenson 1999). In a stricter and more commonly used definition, osteogenesis refers to bone formation by transplanted living cells (Mulliken et al. 1984, Fitch et al. 1997). In this thesis, the latter definition is used.

Osteoinduction: process of recruitment from the surrounding bed of mesenchymal-type cells, which then differentiate into cartilage-forming and bone-forming cells under the influence of a diffusible Bone Morphogenetic Protein (Urist 1980, Stevenson 1999).

Osteoproduction: used here to describe bone proliferation resulting from the combined properties (i.e. osteoinductive, osteoconductive, and/or osteogenic properties) of a grafting material. This term has also been used to describe the properties of class A bioactive materials, such as bioactive glass, that enhance both the proliferation and the differentiation of progenitor cells (Hench 1998, Wilson & Low 1992). As opposed to class B bioactive materials, such as synthetic hydroxyapatite, the properties of which are limited to osteoconduction.

Osteotropism: biodynamic effects of hydroxyapatite ceramic in contact with living bone and resulting in new bone formation in direct contact with the surface of the ceramic, without formation of a separating fibrous layer. Also called bonding osteogenesis (Heise et al. 1990, Burwell 1994).

Transplant: refers to a living graft, as opposed to implant (Urist 1980, Burwell 1994).

Xenograft (adjective, *xenogeneic*): transfer of viable tissue from a donor of a different species. Has replaced the term *heterograft* (Gorer et al. 1961).

Xenoimplant: nonviable tissue transferred between individuals of different species.

3.2. ABBREVIATIONS

AATB:	American association of tissue banks
AIDS:	Acquired immune deficiency syndrome
AW-GC:	Apatite and wollastonite glass ceramics
BIA:	Bone-inducing agent
BMP(s):	Bone morphogenetic protein(s)
CaO:	Calcium oxide
Coll:	Collagen
CRG:	Controlled release glass
CT:	Computed tomography
EDTA:	Ethylenediaminetetraacetic acid

DBM:	Demineralized bone matrix
HA:	Hydroxyapatite
HIV:	Human immunodeficiency virus
kDa:	kiloDalton
KGy:	kiloGray
MAR:	Mineral apposition rate
MTC:	Metacarpal bones
MTT:	Metatarsal bones
Na_2O:	Disodium oxide
NSS:	Normal saline solution
OP (s):	Osteogenic protein(s)
PMMA:	Polymethylmethacrylate
P_2O_5:	Pyrophosphate
SiO_2:	Silica
SEM:	Standard error of the mean
TCP:	Tricalcium phosphate
TGF:	Transforming growth factor

4. INTRODUCTION

Over the last decades, a great deal of research has focused on therapies for enhancing bone regeneration. Their clinical applications are indeed very diverse and expanding to follow the evolution of orthopedic surgery. Over 500000 bone grafting procedures are performed every year in the United States alone, and about twice as many are performed in the rest of the world (Clayton Shors 1999). Stimulating bone production may be applied to the management of fractures, nonunions, and osteomyelitis. Other orthopedic indications include limb lengthening, reconstructive procedures following tumor resection, arthrodeses, and osteointegration of joint replacement prostheses. The clinical use of these techniques actually extends beyond the field of orthopedics to include periodontal, maxillofacial and neurosurgeries. Much progress has been made simultaneously with our improved understanding of bone healing, leading to the elaboration of techniques targeting various factors involved in bone regeneration (Figure 1).

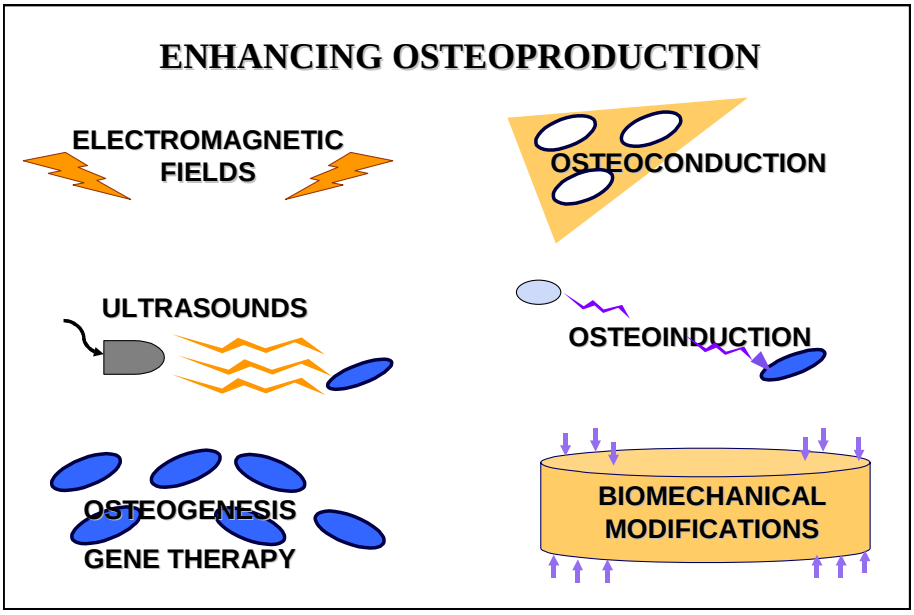


Figure 1: Techniques stimulating bone formation

The importance of the biomechanical environment on bone healing was soon realized, promoting the development of fracture fixation techniques. More recent examples of biomechanical modifications to bone healing include distraction osteogenesis (Ilizarov 1990; Marsh et al. 1997) and axial dynamization of long bone fractures (Egger et al. 1993; Richardson et al. 1995). Other biophysical alternatives to enhance fracture repair involve the use of low-intensity pulsed ultrasonography (Hadjiargyrou et al.1998) and electrical stimulation (Ryabi 1998). Although their mechanisms of action remain somewhat unclear, these techniques have been used in man to

obtain healing of nonunions, accelerate normal fracture healing, and increase healing rates in patients considered at risk for nonunion, such as smokers or diabetics (Cook 1997). Some experimental data are available concerning the effects of these techniques in dogs, and their clinical use is emerging in veterinary orthopedics (Mason & Renberg 2001 a,b). However, among all, bone grafting remains one of the oldest, yet most popular methods to stimulate bone formation.

The first alleged tissue transplantation dates back to the fifth century, with the Miracle of St. Cosmos and St. Damien, according to which the twin brothers resected a diseased limb from a churchwarden and replaced it with the distal extremity of a Moor deceased the same day (Mankin et al. 1983; Hanson & Markel 1992). However, Job van Meekeren was the first to document the use of bone graft in 1668. This Dutch surgeon used a piece of canine skull to replace a skull defect in a Russian nobleman. The operation was successful but resulted in the excommunication of the patient by the church (Boer 1988). It was not until the nineteenth century that the clinical usefulness of bone grafting was recognized. Merrem reported on bone transplants in 1810, and von Walther performed the first recorded human autogenous bone graft in 1820. While Ollier, in 1867, formulated the scientific principles of bone grafting based on his experimental work in France (Ollier 1867; Schweiberer et al. 1989), MacEwen, a student of Lister, performed a successful human bone allograft under aseptic conditions in 1878, in the Infirmary of Glasgow (Chase & Herndon 1955). In the early 1900s, the extensive research performed in Berlin by Axhausen (Burwell 1994) on the transplantation of periosteum, marrow, and bone in rats, rabbits, and dogs provided a bedrock of knowledge that even today remains largely unchallenged. Barth was the first to describe the process of new tissue invading along channels created by invasive blood vessels or along pre-existing channels in the transplanted bone (Barth 1893). This process was called “schleichender ersatz”, a term literally translated by Phemister in 1914 as “creeping substitution”, to describe a dynamic reconstructive and healing process for bone transplantation (Burchardt 1983). In 1911, Albee, a Professor of Surgery in the New York Postgraduate Medical School, initiated the use of living bone grafts as internal splints and reported on thousands of clinical successes a few years later (Albee 1923).

Bone transplants are now routinely used in human and veterinary orthopedics to promote fracture healing, enhance joint fusion, and repair bone defects. Fresh autogenous grafts (Figure 2) are still considered a “golden standard” to which other osteoproductive agents are compared.

Figure 2: Fresh autogenous cancellous graft harvested from the medial proximal tibia of a dog.

Indeed, autogenous bone is the most effective material in promoting rapid healing since, compared with other materials, it provides viable cells while avoiding immune reactions and disease transmission (Fitch et al. 1997). In small animals, fresh autogenous cancellous bone can be harvested from the iliac crest, medial proximal tibia, and proximal humerus, depending on the location of the surgical site. The most common site for collecting cancellous autograft in man is the iliac crest. This procedure is associated with a morbidity rate approximating 25% in humans, with major complications, such as herniation or subluxation of the hip, reported in 3- 4 % of patients (Younger & Chapman 1989; Damien & Parsons 1991). Other complications associated with the collection of autogenous bone include pain, sepsis, stress fractures (Figure 3), intraoperative blood loss, increased surgical time, and limited supply (Damien & Parsons 1991; Ferguson J 1996). Another shortcoming of autogenous cancellous graft consists in its lack of biomechanical strength, which precludes its use as a structural graft.

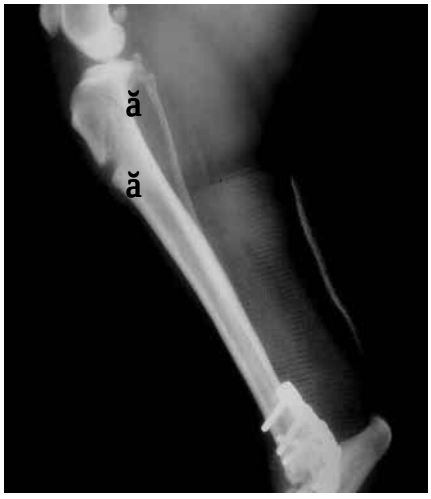


Figure 3: Postoperative fracture of a canine proximal tibia *(arrows). Fresh autogenous cancellous graft had been harvested from the proximal tibia for a tarsal arthrodesis. A splint was placed around the distal limb to support the tarsus (Courtesy of JK Roush).*

To palliate these limitations, the use of allogeneic bone has become popular. Bone can be harvested from live donors (femoral heads during primary hip arthroplasty in man) or cadavers, processed and stored. Interest in methods of preserving and storing bone originates from the experimental work of Bauer who, in 1910, showed that bone refrigerated for as long as three weeks could be successfully transplanted in dogs (Bauer 1910). Bone banking techniques for human use were subsequently developed by Bush and Wilson during World War II in New York and about 30 years later in veterinary orthopedics (Johnson 1988; Burwell 1994). Human autogenous cancellous and cortical bone is now available, allowing surgeons to select the type and exact dimensions of implant required at any time. Canine bone and connective tissue allografts have recently become commercially available in the United States. In most other countries, however, the inconvenience of collection, processing, and storage with a limited shelf life make bone banking impractical even in large referral practices. Nevertheless, segments of cortical bone constitute the most common type of allograft in veterinary orthopedics (see section 5.1.1.). These are used as structural grafts to reconstruct severely comminuted fractures and in limb salvage procedures after tumor excision (Alexander 1983; Larue et al. 1989). The

incorporation of these grafts is slow and incomplete, increasing the risks of fatigue fracture (Henry 1981, Friedlander 1987; Johnson AL et al. 1992; Stevenson 1999). In dogs, infection rates as high as 40% in limb salvage procedures have affected interest in this type of graft (Dernell et al. 1998; Straw et al.1996). Similar complications in man have stimulated the search for alternative strategies to cortical allografting (Mankin et al. 1984; Thompson et al. 1993; Dick & Strauch 1994;). An example involves use of biomechanical forces to stimulate bone regeneration (Figure 1): the reconstruction of large defects can now be achieved by distraction osteogenesis and bone transport techniques. Comparable trends are currently emerging in the veterinary community (Elkins et al. 1993; Lesser 1994; Tommasini Degna et al. 2000).

Although the development of bone graft substitutes and other alternative techniques to stimulate bone healing has gained tremendous popularity over the last two decades, interest in this field has been around much longer. Toward the end of the 19th century, some surgeons focused on bone-derived materials, while others experimented with synthetic agents. Indeed, the first reported use of demineralized bone dates back to 1889, when Senn used xenogeneic, decalcified bone to repair canine skull defects (Senn 1889; Covey & Wright 1992). Three years later, Dreesman obtained healing in six of nine bone defects implanted with plaster of Paris (Peltier 1961; Damien & Parsons 1991). These two protagonists set the scene for the directions taken in the development of grafting materials: bone-derived versus synthetic, osteoinductive versus osteoconductive agents.

Urist pursued Senn's work developing processing techniques for bone. Investigators before him used ethylenediamine tetra acetic acid (EDTA) in the bone demineralization process, which failed to neutralize endogenous neutral proteases, inactivating some of the growth factors within the bone matrix. In 1965, Urist replaced EDTA with hydrochloric acid and described ectopic bone formation following implantation of demineralized bone matrix in rodents (Urist 1965). This served as the basis for the theory of "autoinduction", which, in 1979, led to the isolation of an osteoinductive, low-molecular-weight protein from insoluble bone matrix, named "bone morphogenetic protein" or BMP (Urist 1980; Urist et al. 1983). In an attempt to reduce antigenicity and avoid irradiation, Urist also produced an autolyzed, antigen-extracted allogeneic bone (AAA bone), with preserved osteoinductivity and a mechanical strength compatible with its use in vertebral fusion (Urist 1981a,b). The concept of delivery systems for BMPs originates from Sampath and Reddi, who demonstrated in 1981 that neither the postextraction bone matrix nor the residue alone induced ectopic bone formation in rodents, but that the osteoinductive properties were totally restored by combining both (Sampath & Reddi 1981). They also introduced the homology of osteoinduction in 1983, showing that proteins extracted from human, monkey, rat, and bovine extra cellular matrix could all induce bone formation when combined with inactive allogeneic bone matrix (Sampath & Reddi 1983).

Further analysis of the BMP fraction contained in the extra cellular bone matrix led to the identification of several distinct proteins that, either alone or in combination with other regulatory molecules, could induce bone formation (Wolfe & Cook 1994). In 1987, Sampath and colleagues isolated an osteoinductive extra cellular matrix-associated protein from demineralized bovine bone matrix, which seemed 1000 times more potent than BMP (Sampath et al. 1987). This protein was named "osteogenin". A year later, Wang et al. purified BMPs for the first time, isolating three polypeptides of 16-, 18- and 30-kDa molecular weight from bovine bone. These would eventually be named BMPs 1-3 (Wang et al. 1988). While purification decreased the

likelihood of adverse effect associated with immunogenic impurities, the process was laborious and inefficient. Synthetic osteoinductive agents were therefore developed as soon as purification of osteoinductive proteins reached sufficient quantity and homogeneity to provide amino acid sequence data. Human complementary DNAs could then be isolated and cloned, and the osteogenic protein gene product recovered from host cell-conditioned media. A significant step in characterization of BMPs came in 1988, when Wozney and coworkers isolated human complementary DNA clones corresponding to bovine BMP-1, BMP-2A (BMP2), and BMP3 (Wozney et al. 1988). All BMPs except BMP-1 belong to the TGF-β superfamily (see section 5.2), a group that countains more than 15 molecules and continues to grow as new members are identified. At least 10 BMPs have been identified, but two of these proteins have been particularly well described in man: recombinant BMP-2 and –7 (OP-1) (Wang et al. 1988, 1990; Wozney et al. 1988; Chen et al. 1991; Yasko et al. 1992; Cook et al. 1994, 1995; Geesink et al. 1999; Mason & Renberg 2001). Their properties have been studied in several animal experiments, and the most advanced human clinical trials have focused on these two growth factors (Yasko et al. 1992; Cook et al. 1994, 1995; Kirker-Head et al. 1996; Rowley 2001). Geesink and coworkers recently validated the efficacy of BMP-7 in critically sized fibular defects created in patients undergoing tibial osteotomy (Geesink et al. 1999). In this double-blind, randomized prospective human clinical trial, 5/6 defects filled with decorticated bone or BMP-7 (with collagen) healed, whereas none of the defects left empty or filled with collagen alone healed. Such studies are few as the technology is fairly recent and controlled clinical evaluations are difficult to perform. Further randomized prospective clinical trials are therefore warranted to justify routine use of osteoinductive agents in human orthopedics (Ladd & Pliam 1999).

Clinical use of osteoconductive materials, in contrast, has gained much popularity. In fact, their use dates back further than that of osteoinductive agents, as most allogenic bone grafts (undemineralized) are deprived of viable cells and therefore act only as osteoconductive supports. The major contribution to the field of osteoconduction made in the 20th century consisted of the development of synthetic agents. Calcium sulfate (plaster of Paris) was one of the first materials investigated as a synthetic osteoconductive bone graft substitute. Based on experimental data in dogs and clinical experience in humans, Peltier eventually concluded that plaster of Paris is easy to use, inexpensive, readily available, stable for filling cavities in bone, and does not inhibit bone healing (Peltier 1961). These findings were later confirmed in canine femoral defects (Elkins & Jones 1988). Calcium sulfate is produced by calcining natural gypsum at temperatures of 110 - 130 °C, which removes 75% of the water and results in crystals of irregular shape. Because of its natural origin, gypsum may contain impurities, such as iron, magnesium, strontium, lead, and other heavy metals, prior to processing (Damien 1991). Concerns were raised over the potential toxic effects, poor osteoconductive properties, fast resorption and poor mechanical strength of plaster of Paris, and its clinical use declined as bioactive ceramics gained popularity (Damien 1991). The interest in calcium phosphate as hard tissue implants initially evolved around the hypothesis that release of calcium ions would stimulate osteogenesis (Leriche & Policard 1928). Two main groups of ceramics (see section 5.3.1.) were evaluated: hydroxyapatite (HA) and tricalcium phosphate (TCP). Biodegradability became an issue; TCP was found to resorb within days to weeks *in vitro* and *in vivo*, whereas HA resorbed slowly (within years), if at all (Klein et al. 1983). To obtain a balanced resorption, one option therefore consisted of mixing both materials, anticipating that TCP would provide minerals to feed bone cells while HA would act as a scaffold. This approach has been adopted by many research groups in Europe and in the United

States. Another approach lead to developing macroporous ceramics. The importance of porosity for resorption and effective bone growth into ceramic structures was outlined by Klawitter in 1971 (Klawitter & Hulbert 1971). One way of obtaining interconnective pores of at least 100µ consisted of using coral as an implant. The interest in scleractinian corals, such as *Gonoporia* and *Gorites*, was triggered by their macroscopic porous architecture, approaching that of human cancellous bone. Moreover, the use of coral pore structures was taken a step further with the development of the replamineform technique (meaning replicated life forms), a hydrothermal exchange method for producing ceramic replicas of the coral structure (White et al. 1972). The advantage of the coralline hydroxyapatite produced with this method over coral is that it is devoid of any residual organic matter. The use of coral as a source of hydroxyapatite relies on natural supplies, which may eventually become insufficient to satisfy a growing market. Although tons of hydroxyapatite are mined from the earth each year as a source of phosphate ion, researchers have spent the last 20 years experimenting with hydroxyapatite to attain a level of purity and consistency compatible with medical use (Technical monograph 1986; Burwell 1994). Most of the ceramics produced for clinical use are manufactured, and their properties can be adjusted to match specific applications. The resorption rate of HA can be accelerated by increasing porosity, but the resultant decrease in biomechanical strength prevents its use as a structural graft. Therefore, research interest has focused on improving the biomechanical strength of materials while maintaining osteoconductivity, bioactivity, and resorbability (Oreffo & Triffitt 1999). Wollastonite-reinforced glass ceramic has been reported to be biomechanically superior to TCP-HA composites (Kokubo 1993), but bioactive glasses are generally considered too brittle for use in large bone defects (Damien & Parsons 1991; Virolainen et al. 1997). Another alternative relies on toughening techniques for ceramics, such as designing ceramic-ceramic composites incorporating minor amounts of metastable, dispersed second-phase particles (Kuo & Kriven 1998, Gulgun et al. 1999). However, none of these materials has been evaluated *in vivo*.

For many years, developments in the field of biomaterials generally involved trial-and-error experiments (Hench 1998). This approach has often been successful, and over the last 30 years, more than 40 different ceramic, metal, and polymeric materials have been used to replace, repair, or augment more than 40 different parts of the human body (Hench & Wilson 1993; Ratner et al. 1996). This rapidly evolving technology may seem confusing and may challenge the ability of clinicians to formulate an informed opinion on emerging therapies. Orthopedic surgeons should objectively review any experimental and clinical data compiled by manufacturers and associated research centers to convince themselves of the safety and efficacy of new products. Unfortunately, this exercise can be time-consuming and requires a good understanding of research protocols. The wide therapeutic range now offered poses yet another dilemma for clinicians. While it provides more freedom to optimize treatment modality for individual cases, it also complicates the decision-making process for orthopedic surgeons. A rational attitude towards solving this issue is based on an assessment of the factors affecting bone healing (Figure 4).

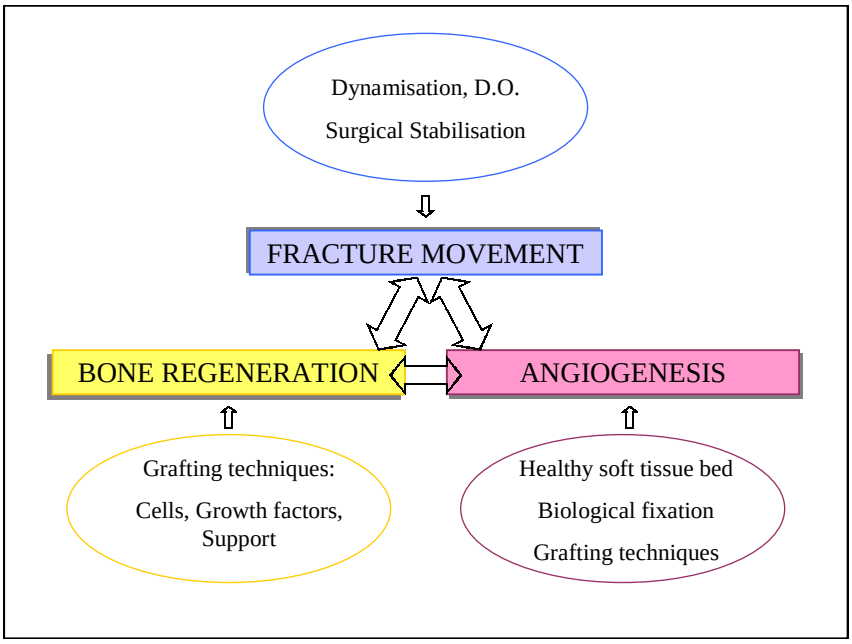


Figure 4: Diagram derived from Marsh and Li's triad of interrelationships in the regenerative repair of fractures *(Marsh & Li 1999).*
D.O.: Distraction osteogenesis.
Support: gap filler and network for osteoprogenitor cells.

Understanding which factor(s) are deficient in a given patient determines the criteria for selecting a therapeutic strategy. If grafting is the answer, a good knowledge of the grafting agents available will guide the ultimate choice. Table 1 summarizes the properties of grafting materials relevant to an appropriate selection. In difficult cases, several contributing factors can be identified and a combination of therapies required. For example, highly comminuted fractures in smokers may justify the use of cancellous allograft as an osteoconductive gap filler, mixed with a gel of demineralized bone matrix to provide growth factors. In postirradiation patients, this combination can be further augmented by an autogenous bone marrow aspirate to increase the local population of mesenchymal stem cells (Redfern 2001). Researchers are currently investigating alternative techniques to develop safe osteogenic grafts. Mesenchymal cells have experimentally been harvested from recipients (via muscle or mesenchymal tissue biopsy, or bone marrow aspirate) and allowed to multiply *ex-vivo* (Kadiyala et al. 1997, Scaduto & Lieberman 1999). DNAtransfer to these cultured cells can then occur via viral gene vectors (e.g. adenovirus, adeno-associated virus, and herpes virus) to stimulate their synthesis of growth factors such as BMP-2 (Evans & Robbins 1995). This technology can be applied to bone formation, but also to stimulate the repair of cartilage, osteochondral defects, and tendons (Evans & Frisbie 2000; Imhoff & Martinek 2001).

As a simple answer rarely addresses a difficult problem, it is unlikely that one grafting agent will eventually be found to optimally resolve all orthopedic conditions where bone formation is challenged. Instead, individual types of grafting materials are likely to improve in their biomechanical and biological performance as well as diversify and intertwine with each other to better answer specific issues. A thorough and long-term evaluation of new biomaterials is indicated to verify their superiority over clinical controls and prevent side-effects such as transmission of pathogens or unpredictable resorption of the implant and newly formed bone.

"Le primum non nocere"

Table 1: Properties of grafting materials.

Biomechanical Structural Nonstructural	**Biological properties** Osteogenesis Osteoinduction Osteoconduction Composites
Origin Synthetic Natural Bone-derived Others (e.g.: Coral)	**Sterilization** Aseptic collection and processing Irradiation: Dose and temperature (Hamer et al. 1999) Chemical: Demineralization process (Scarborough et al. 1995) Ethylene oxide (veterinary only)
Presentation Massive Segments, blocks, or struts Chips Granules Particle size Particle size distribution Putty, paste, gel Powder Fibers, flex (flexible grafts)	**Availability** **Cost**

5. REVIEW OF THE LITERATURE

5.1. LIMITATIONS OF ALLOGRAFTS

5.1.1. Processing and biomechanical properties:

The most common use of cortical allografts in veterinary medicine has been in highly comminuted diaphyseal fractures (Figure 5) (Phillips et al. 1988). Cortical bone grafts and implants have also been used to replace segments of bone after tumor excision (LaRue et al. 1989), lengthen bones, and treat selected nonunions (Alexander 1983).

Figure 5: Use of a cortical allogeneic bone segment to reconstruct an atrophic nonunion fracture in a canine femur.
Arrows point at the host-graft junctions.

In all of these cases, cortical allografts must retain biomechanical properties so that the repaired bone can withstand forces generated during weight bearing. Biomechanical failure of cortical allografts has been documented (Mankin 1984; Berry et al. 1990; Wagner et al. 1994). Use of fresh grafts ensures integrity of biomechanical properties, because the bone is unaltered structurally, but may generate an inflammatory response from the recipient (Friedlander 1983), especially in the event of major histocompatibility mismatches (Stevenson et al. 1991).
Long-term preservation of bone has been developed to provide grafts at any time and in a variety of sizes. Multiple methods for sterilization and storage of cortical bone, including irradiation, ethylene oxide sterilization, freezing, and freeze-drying, have been described in people (Tomford et al. 1987; Ivory & Thomas 1993; Tomford & Mankin 1999). Some of these techniques alter its biomechanical properties and make it unsuitable as a structural graft (Triantafyllou et al. 1975; Pelker et al. 1984; Johnson et al. 1987; Roe et al. 1988; Itoman & Nakamura 1991, Boyce et al. 1999). For example, gamma irradiation is the most popular sterilization technique of human bones harvested from cadavers. A dose above 30 kGy is currently recommended to eradicate the

human immunodeficiency virus (HIV). The adverse effects of such a high dose of radiation on bone graft are well established with respect to mechanical performance (Pelker & Friedlander 1987). A common method for banking cortical bone in veterinary medicine includes clean collection on cadavers and freezing after ethylene oxide sterilization. This sterilization method is not recommended in human orthopedics because residual gas may remain within transplanted tissue and cause irritation (Norman-Taylor & Villar 1997). Sterile collection and freezing under various conditions have been used in veterinary surgery to avoid side-effects of sterilization (Denny 1985; Johnson 1991). Freezing does not seem to alter the biomechanical properties of cortical bone if surface hydration of the tissue is preserved (Bright 1983). Addition of sodium chloride or lactated Ringer's solution has been suggested to protect frozen bone from dehydration (Brinker et al. 1990; Laforest et al. 1991), but its effects on the biomechanical properties of frozen bone have not been evaluated.

5.1.2. Health and safety issues:

The major disadvantage associated with sterile collection and freezing of bone involves transmission of pathogens. Experiments have demonstrated the ability of fresh-frozen connective tissue allografts to transmit the feline leukemia retrovirus (Nemzek et al. 1994). This risk is especially relevant in man, where diseases transmitted in bone allografts have included hepatitis B, hepatitis C, and acquired immnune deficiency syndrome (AIDS) (Tomford & Mankin 1999). Screening of donors and removal of blood products from bone allografts have helped reduce this problem. According to the American Association of Tissue Banks (AATB) records, only two cases have been linked with documented transmission of AIDS since identification of the virus (American Association of Tissue Banks 1993; Gazdag et al. 1995). Both cases involved transplantation of unprocessed, fresh-frozen allografts, with the last case dating back to 1985 (Boyce et al. 1999). However, any grafting material containing human tissue carries a potential for disease transmission. The emergence of new pathogens, possibly more resistant to the sterilization techniques currently used prior to banking, has also raised public concern over the use of allografts.

5.1.3. Shortage of supply:

More than 15000 bone allografts are used every year in the United States alone (Tomford & Mankin 1999). Most originate from cadavers, although they may occasionally be harvested from live donors. For example, the most common source of corticocancellous allografts utilized in revision hip arthroplasties consist of femoral heads excised during a primary procedure. Over 800000 total hip replacements are performed annually worldwide, with approximately 150000 occurring in the United States (Behairy & Jasty 1999). Nonetheless, the supply may run short. For instance, the current harvest of approximately 1700 femoral heads per year in Scotland is expected not to meet the future demand for revision surgery of the hip (Galea et al. 1998). This imbalance results from a couple of reasons: hip arthroplasty has become increasingly popular and is now applied to treat a greater variety of diseases (such as immune arthrites). In these cases, the longevity of the implant is decreased and early revision is expected. As the number of implants in place for more than 10 years increases, so does the number of revisions required: revision hip arthroplasties now represent about 10-20% of the case load of joint replacement surgeons (Bourne & Crawford 1999).

To palliate the limitations of autografts and allografts, a great deal of interest has focused on the development of bone substitutes, which may be broadly classified as osteoinductive or osteoconductive.

5.2. OSTEOINDUCTIVE BONE GRAFT SUBSTITUTES

Osteoinductive bone graft substitutes initiate and stimulate the differentiation of undifferentiated mesenchymal cells into osteoprogenitor cells (Urist et al. 1983; Forell & Straw 1993). One strategy for developing a bone-inducing agent is to identify a single purified molecule, which could ultimately be confirmed as the agent of choice for clinical promotion of bone healing and regeneration (Anderson 1994). Investigators have subsequently attempted to isolate, purify, partially sequence and then clone the cDNAs for all presently known bone morphogenetic proteins (BMP) (Wozney et al. 1988, Cook et al. 1994, Kirker-Head 1995). At least 10 BMPs have been identified (Table 2) but two of these proteins have been particularly well described in man: recombinant BMP-2 and –7 (OP-1) (Mason & Renberg 2001).

Table 2: Members of the Transforming Growth Factor Beta superfamily of genes.
All Bone Morphogenetic Proteins belong to this group except BMP-1. Members of each subfamily share 50-90% genetic-sequence homology, and 30-40% genetic-sequence homology with members of other subfamilies. Derived from Reddi 1994 and Einhorn 1995.

Transforming growth factor-beta subfamily	**Bone morphogenetic subfamily**
Transforming growth factor-beta 1 Transforming growth factor-beta 2 Transforming growth factor-beta 3 Transforming growth factor-beta 4 (chicken) Transforming growth factor-beta 5 (Xenopus)	Bone morphogenetic protein-2 (or bone morphogenetic protein-2A) Bone morphogenetic protein-3 (or Osteogenin) Bone morphogenetic protein-4 (or bone morphogenetic protein-2B) Bone morphogenetic protein-5 Bone morphogenetic protein-6 (or Vgr 1) Bone morphogenetic protein-7 (or osteogenic protein-1 or OP-1) Bone morphogenetic protein-8 (also known as OP-2) Bone morphogenetic protein-9 Bone morphogenetic protein-10 Decapentophegic gene (Drosophilia) Growth and differentiation factor-1 Vegetal pole-derived gene (Xenopus) Drosophilia 60A
Inhibitin-activity subfamily Inhibitin-Ŭ Inhibitin-βA Inhibitin-βB Müllerian Inhibitin substance	

However, there is increasing evidence that bone repair may be optimally enhanced by a combination of agents (Anderson 1994; Anderson et al. 1995). Therefore, a second strategy for development of bone-inducing agents is to identify an optimal mixture of bone growth agents, either concocted from purified agents, or generated biologically as the product of a tissue or a

cultured cell. The tissue most commonly used as a source of partially purified bone-inducing factors has been decalcified bone (Table 3) (Bolander & Balian 1986; Urist et al. 1987; Hopp et al. 1989; Johnson EE et al. 1992). Demineralized bone matrix (DBM) is commercially available as several preparations, differing in the processing technique and composition. For example, carriers for demineralized bone matrix include glycerol, pluronic-F127 copolymer, porcine gelatin, hyaluronic acid, corticocancellous chips, collagen, and a mixture of calcium sulfate and methylcellulose (Abjornson Manitta 2001). Manufacturers claim that this variation in formulation may affect the osteoinductive properties of the final product (Russel 2001).

Table 3: Examples of osteoinductive agents under consideration, or currently used for clinical applications.

Product name	**Content**	**Comments**
BONE DERIVED		
AAA Bone	Human chemosterilized autolized antigen extracted bone	Described by Urist in 1981 Structural and delivery system for hBMP in nonunions (Johnson 2000)
Grafton (Osteotech Inc., USA) previous DBM gel	human DBM, available since 1991	Glycerol as a carrier Gel, putty, flex, fibers and crunch (fibers and cubes)
Osteofil (Sofamor Danek Group Inc., USA)	human DBM: 24% + 17% porcine gelatin as carrier, in aqueous solution	Moldable, hardens to a rubber-like consistency at body temperature. Lack of clinical data (Ladd 1999)
DynaGraft (GenSci, Canada)	human DBM	Lack of clinical data (Ladd 1999)
Opteform (Exactech Inc, USA)	Compacted corticocancellous human bone chips and human DBM+porcine gelatin+water	Moldable putty Lack of clinical data (Ladd 1999)
Canine DBM (Veterinary Transplant Services, USA)	Demineralized bone matrix	Cortical powder, with or without cancellous chips Veterinary use
COMPOSITES		
rhBMP-2 (Genetics Institute, USA)	Synthetic BMP with an absorbable collagen sponge as a carrier	Undergoing clinical trials: Tibial callotasis (Eyres 2001)
rh-BMP-7 (Ceative biomolecules, USA)	Synthetic BMP with collagen	Undergoing clinical trials: Osseous defects (Geesink 1999)
Osteoconductive grafts (see table below) mixed with demineralized bone matrix or autogenous bone marrow		

The major limitation to the clinical use of such preparations is the very low concentration of bone morphogenetic proteins in whole bone (Figure 6): about 1 mg of pure BMP can be extracted from 1 kg of bovine bone (Wozney 1993). This considerably increases the cost of extracting a native bone-inducing therapeutic agent and does not eliminate the risk of disease transmission.

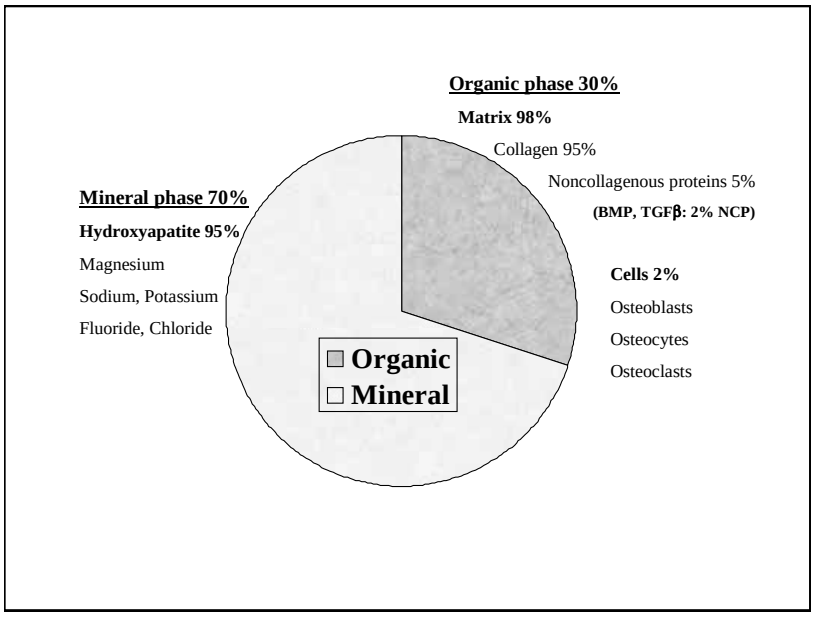


Figure 6: Composition of bone.

Extraction of a natural osteoinductive agent from cultured cells may be a less expensive alternative for the unlimited production of a bone-inducing agent. A Bone-inducing agent (BIA) extracted from a cultured human osteosarcoma cell line (Saos-2) and has been found highly osteoinductive when implanted in the skeletal muscle of athymic mice (Anderson et al. 1992). Ten milligrams of BIA extracted from Saos-2 cells (mixed with bovine collagen) promotes early osseous union of an internally fixed diaphyseal femoral nonunion in immunocompetent rats (Hunt et al. 1993). In this study, an equal amount of bovine collagen was mixed to the BIA to act as a slow-release protein delivery system. Osteoinductive agents are always combined with a carrier, which usually consists of an osteoconductive biomaterial.

5.3. OSTEOCONDUCTIVE BONE GRAFT SUBSTITUTES

Osteoconductive materials provide a passive scaffold onto which osteoprogenitor cells can lay new bone. Osteoconductive agents (Table 4) include ceramics, collagen, porous metals, polyglycolic and polylactic acid polymers, and bioactive glasses (Cornell 1999). They are usually

too brittle to bridge large bone defects but are used as nonstructural fillers in maxillofacial, neurologic and orthopedic surgery. They can also function as carriers for growth factors and antibiotics, and may present as blocks, fibers, granules, powders, gels, and sprays, depending on the application. The properties of osteoconductive grafts will therefore be determined by their physical presentation as well as other factors, such as chemical composition, processing, granulometry, and interconnective porosity (Table 1).

Table 4: Some commercial forms of osteoconductive biomaterials.

Product name	Content	Comments
BONE DERIVED		
Kiel bone (Surgical University Clinic of Kiel, Germany)	Bovine bone deproteinized by hydrogen peroxide and defatted with ether	Historical use in 1960s
Opteform	Compacted corticocancellous bone chips	Contains gelatin and water
Bio-oss	Sintered bovine bone	
Oxbone	Sintered bovine bone	
Endobon (Merck KGaA, Germany)	Sintered bovine cancellous	Blocks Use approved in Europe, not USA.
Surgibone (Unilab Inc, UK)	Processed mature bovine bones	Contain mainly HA and 20-29% proteins
Lubboc (Transphyto, France)	Processed young bovine cancellous bone	Blocks, plates, fragments
Pyrost	Sintered bovine bone	Contains 93% HA, stronger than Collapat. Potential structural graft
Osteoplant (BioTeck, Italy)	Equine irradiated demineralized bone matrix	Blocks, granules, flex Cancellous or cortical origin
Canine bone allograft (Veterinary Transplant Services, USA)	Cancellous or cortical bone	Whole bones, sections, blocks, wedges, struts, cancellous chips Veterinary use
Osteomin (Pacific Coast Tissue Bank, LA, USA)	human bone ash: Microporous HA	Particulate
Banked human allograft	Cancellous, cortical, corticocancellous, or osteochondral bone	Sections, blocks, wedges, struts, chips, particulate, powder Properties vary with processing

HYDROXYAPATITE		
Biocoral (Inoteb, France)	Natural mineral skeletons of scleractinian corals	Manufacturer recommends augmentation with autogenous bone marrow
ProOsteon (Interpore Int., USA) previous name: Replam Hydroxyapatite-Porites or RHAP	Replaniform coralline macroporous HA 200: derived from Porites porites (pore size 190-230 µm) 500: derived from Porites Goniopora (larger pores) R: Resorbable	Particulate or block Brittle. Radiopacity impedes assessment of healing. Slow resorption R-form produced by premature termination of the thermochemical exchange
Calcitite (Calcitek, USA)	Dense crystalline HA	Granules, diameter: 400-800µm
Periograf (Cooke-Waite Laboratories, USA)	Dense crystalline HA	
Ceros 80 (Straumann Ltd, UK)	Dense polycrystalline HA	Particules with very little to no resorption *in vivo*
Osprovit (Cerasiv, Germany)	Porous HA (pore size: 200-600 µm)	Oral and maxillofacial surgery
OrthoMatrix HA-SYSTEM	Includes HA-500, HA-1000, and HA-2000, with particle diameter ranging from 240 to 2000 µm	Particulate, oral, and craniofacial surgery
Friabone, Algipore and Frialit (Friedrichsfeld, Germany)	HA	
Biosorb	HA	
Cerapatite (Ceraver Osteal, France)	HA	
OsteoGraf (CeraMed, USA)	Crystalline HA	AR or P: dense N or M: microporous
OsteoGen (Impladent, USA)	Crystalline HA	Particulate
TRICALCIUM PHOSPHATE AND CEMENTS		
Ceros 82	β-TCP, porosity varies to adjust resorption between 6 and 12 months	Lower compressive strength than Ceros 80
Calciresorb (Ceraver Osteal, France)	Porous TCP	Periodontal applications
Synthograf (Miter, USA)	Small size and dense TCP	Periodontal applications
Augmen (Miter, USA)	Larger size and dense TCP	Periodontal applications
BoneSource (Howmedica, USA)	Calcium phosphate	Putty, maxillofacial surgery
Alpha BSM	Calcium phosphate	Moldable putty, maxillofacial surgery
Orthocomp	Calcium phosphate	Cement

Norian Skeletal Repair System (SRS) (Norian Corp., USA), formely Superbone	Powdered calcium phosphate and calcium carbonate mixed with a solution of calcium phosphate prior to use	Injectable cement, augmentation of fracture repair. Hardens in 10 mn, generating negligible heat. Support in compression, weak in torsion and shear
E-TEX α-BSM (E-TEX, USA)	Calcium phosphate	Injectable cement, dental applications as a void filler
Vitagraft (Orthovita, USA)	Calcium phosphate	Moldable cement
OSTEOCONDUCTIVE COMPOSITES		
Triosite (Zimmer Europe Ltd, UK)	60% HA, 40% TCP	Also called MBCP (macroporous biphasic calcium phosphate) or BCP
Ostilit (Stryker Howmedica Osteonics, UK)	80% TCP, 20% HA, not macroporous	Granules and blocks nonstructural graft
BoneSave (Stryker Howmedica Osteonics, UK)	80% TCP, 20% HA, pore size: 400-600μm	Granules, stronger than Ostilit, for use as a void filler and in impaction grafting
Healos	HA coated bovine collagen	Sponge
Collagraft (Zimmer Inc, USA), formerly Colhap	Bovine collagen, 60%HA and 40% TCP, pore size: 300-700μm	Granules and strips Requires augmentation with aspirated marrow
Collapat (Ostobalag, Switzerland)	Collagen + crystals (<20μm diameter) of 90% HA and 10%TCP	Collagen: commercial preparation of pig skin (Pentapharm) Nonstructural applications
Cerapatite-collagen (Ceraver Osteal, France)	HA-Collagen	Nonresorbable
Calciresorb-collagen (Ceraver Osteal, France)	TCP-Collagen	Resorbable
Biofibre (Norian Corp., USA)	Spherulite formulation of calcium phosphate crystals mixed with collagen	
TITANIUM CYLINBERS AND FIBER METAL RODS		
Modular Segmental Spinal Instrumentation or MOSS (Biedermann Motech GmbH, Germany)	Titanium-mesh cylinder	Vertebral substitute
BIOACTIVE GLASSES (see table below)		
CARBON FIBERS		
OTHERS		
Osteoset (Wright Bio-Orthopedics, USA)	Calcium sulfate	Pellets, resorbed in 6-8 weeks, no structural support
HTR synthetic bone (Bioplant, USA)	Polymer: Microporous composite of PMMA, calcium hydroxide, polyhydroxylethylmethacrylate	HTR: hard tissue replacement Seemed to hinder healing Historical use

5.3.1. Ceramics

Synthetic hydroxyapatite, tricalcium phosphate, and combinations of the two are the prevailing bone graft substitutes (Behairy & Jasty 1999). The mineral phase of bone represents approximately 60% to 70% of its dry weight (Figure 6) and consists of carbonated, calcium phosphate apatite (Posner 1985). Ideally, a bone graft substitute would have a similar mineral composition and structure. This was one of the reasons why calcium phosphate substrates supplanted calcium sulfate compounds, such as plaster of Paris (Tay et al. 1999). Klawitter (Klawitter & Hulbert 1971) established the need for ceramics to present pores of at least 100 µm to allow bone ingrowth. Porous hydroxyapatite (HA) and tricalcium phosphate (TCP) are typically produced by isostatic compaction of calcium phosphate powder with naphtalene. The naphtalene is subsequently removed, creating a porous structure that is then sintered (Cornell 1999). Biodegradation of TCP and HA relies on cell-mediated mechanisms and dissolution, both of which are related to implant surface area (Holmes 1994). Porosity, as well as particle size, will therefore affect the degradation rate of the compound and the biomechanical properties of the implant. Thus, it is crucial to differentiate between dense and macroporous forms of hydroxyapatite (Burwell 1994). The latter has a higher resorption rate but is mechanically weaker. Because TCP dissolves more rapidly than HA (Tay et al. 1999), it is also possible to adjust the resorption kinetics of composite materials by varying the ratios of hydroxyapatite and tricalcium phosphate (Behairy & Jasty 1999). Although TCP/HA mixtures have been evaluated in bone defects (Gatti et al. 1990; Frayssinet et al. 1993), further studies are needed to determine the ideal mixture to be used as an impacted, idealised morselized aggregate in revision hip arthroplasty.

5.3.2. Bioactive glasses

A potential advantage of bioactive glass over HA and TCP is a superior biomechanical strength, while simultaneously retaining the ability to form a strong chemical bond with bone. According to Yamamuro, glass ceramics containing apatite and wollastonite (AW-BC) synthesized since 1980 at Kyoto were stronger than human cortical bone and bonded with living bone within 8-12 weeks (Yamamuro et al. 1987; Kokubo 1993). This material was successfully used as vertebral prosthesis following spinal tumor removal in four patients.
Bioactive glasses contain SiO_2 as a network former, which results in slow and incomplete resorption. Controlled release glasses (CRG) are inorganic polymers based on phosphates of sodium and calcium converted into a glassy form. They differ from other traditional bioactive glasses (Table 5) (Fetner et al. 1994; Heikkilä et al. 1995; Hench 1998; Ikeda et al. 1999) in that they do not contain SiO_2. When exposed to tissue fluids, traditional bioactive glasses form a bonding layer of biological hydroxy-carbonate-apatite with an underlying layer of silica gel (Hench & Parschall 1973; Wilson et al. 1993), while CRGs dissolve completely in water and create an acidic environment (Gilchrist et al. 1991).

Table 5: Examples of bioactive glasses available commercially.

Bioglass (American Biomaterials Corp., USA)	Bioactive glass, 45% silica, 24.% calcium oxide, 24.5% disodium oxide, 6% pyrophosphate	Original glass developed by Larry Hench in the 1970s
Consil (Xeipon Ltd, UK)	Bioactive glass	Particulate bioglass for veterinary use
NovaBone (American Biomaterials Corp., USA)	Bioactive glass	Reconstructive procedures and void filler, resorption rate? Limited clinical data
Biogran (Orthovita, USA)	Bioactive glass, narrow particle size range: 300-355 μm	Void filler, dentistry Superior osteoconduction?
PerioGlass (Block Drug Co., USA)	Bioactive glass, particle size range: 90-710 μm	Void filler, dentistry
Ceravital (E.Pfeil & H. Brömer, Germany)	Group of glasses and glass ceramics of various compositions	Experimental use nonstructural grafts for ear surgery.

Controlled release glasses are biocompatible and have been shown to allow rapid growth of viable bone, in a manner similar to autografts (Burnie & Gilchrist 1983; Paul et al. 1983; Burnie et al. 1991). Blocks of bioactive glass alone have been found to be too brittle for use in structural reconstructions (Damien & Parsons 1991; Virolainen et al. 1997). Likewise, uncompacted aggregates of a new controlled release glass, Corglaes®, have been shown to be weaker than morselized bone (Brewster et al. 1999). However, correcting particle size distribution of morselized allografts produced for clinical use in revision hip arthroplasty with small particles of Corglaes® ("idealizing the graft") improved their biomechanical properties (Brewster et al. 1999). CRGs may therefore be considered as potential graft substitutes in revision hip arthroplasty, although the biological properties of impacted aggregates of morselized CRGs warrant further investigation.

5.4. IMPACTION GRAFTING FOR REVISION HIP ARTHROPLASTY

Numerous techniques have been described for revision hip arthroplasty in man, but the outcome still remains less favorable than that of primary arthroplasties (Brewster et al. 1999, Robinson et al. 1999). The endosteal surface surrounding loose femoral stems is often smooth and compromises adequate micro-interlock of the cement with the host (Dohmae et al. 1988; Bourne & Crawford 1999). The loss of bone stock induced by wear particles may also be such that mechanical containment of the implant and cement mantel is difficult to achieve (Berry 1998). One technique to overcome these problems consists of packing morselized corticocancellous allograft around a cemented implant (Figure 7). Impaction grafting in revision hip arthroplasty was first reported for acetabular reconstruction by Sloof in 1984, and was later described for revision of the femoral component by Simon et al. (1991).

Figure 7: Postoperative radiographs of a patient who had undergone bilateral impaction grafting hip arthroplasty. *Note the decreased radiopacity of both proximal femora. On each side, the femoral cortex is reinforced with a metallic mesh and cerclage wires prior to impacting corticocancellous allograft in the canal. The femoral prosthesis is a collarless polished tapered stem (Exeter™) (Courtesy of D Dunlop).*

Early clinical results were encouraging (Gie et al. 1993), but recent studies have reported a high incidence of subsidence and fracture of the femoral stem (Eldridge et al. 1997; Leopold et al. 1999; Knight & Helming 2000). Further evaluation of the mechanical and biological behavior of impacted morselized grafts seems therefore warranted.

Nevertheless, the use of impaction grafting remains particularly appealing for revision arthroplasty in patients with severe osteopenia of the proximal femur (Meding et al. 1997). Potential advantages of this technique include increased interdigitation of the cement with the graft and augmentation of proximal femoral bone stock (Schreurs et al. 1994; Nelissen et al. 1995). However, safety concerns and limited supplies have stimulated the search for synthetic agents to augment or replace allografts. Correcting the particle size distribution of morselized allografts produced for clinical use with small particles of bone or synthetic material ("idealising the graft") has also been shown to improve their biomechanical properties (Brewster et al. 1999). These findings correlate with the particulate aggregate theory, according to which well-graded grafts, with a particle size distribution approaching the ideal logarithmic grading line, resist shear strains better than aggregates of more uniform size. However, the resultant increase in cohesion may affect neovascularization and incorporation of the graft.

5.5. ANIMAL MODELS

Osteoinductive properties of grafts are typically tested in sites void of osteoprogenitor cells, so that any new bone will result from the differentiation and proliferation of undifferentiated mesenchymal cells. Muscle and subcutaneous tissues are the most common implantation sites for osteoinductive bioassays. The latissimus dorsi muscle is an attractive site of implantation because it is easy to expose and offers a large section of muscle of similar thickness. Bone formation is usually detected within two weeks after heterotopic implantation of an osteoinductive agent in athymic mice. In monkeys and dogs, osteoinductive bioassays are usually prolonged to six to eight weeks (Sato & Urist 1985; Miyamoto et al. 1993; Ripamonti 1993) to compensate for their slower metabolic rate compared to rodents (Sato & Urist 1985; Cook et al. 1994).

Osteoproductive properties in general, and osteoconduction in particular, are evaluated in orthotopic sites. Unicortical bone defects are well-established models for evaluation of the biological properties of grafting materials. Unicortical defects have several advantages over non-union models: they do not require internal fixation and allow the evaluation of several local growth factors in the same animal. One of their limitations is related to the size of the defect and the risk of fracture secondary to a stress rising effect. Diaphyseal defects involving 20% of the diameter of the bone decrease the torsional strength by 34% (Edgerton et al. 1990). Whereas previous studies describe the use of diaphyseal unicortical defects measuring up to 9 mm in diameter in sheep (Frayssinet et al. 1993), the metaphysis of long bones can accommodate larger defects (Sartoris et al. 1986; Sauk & Van Kampen 1991). The other advantage of metaphyseal defects over diaphyseal defects is related to the balance between osteogenesis and biodegradation of grafting materials in cancellous bone. Lu et al. (1998) recently compared tissue reactions to calcium phosphate ceramics among cancellous, cortical, and medullar implantation sites in rabbits: medullar sites exhibited stronger immune response and faster biomaterial degradation, whereas osteoproduction was increased within cortical sites. Cancellous sites had intermediate activities and are therefore recommended as models for evaluation of the properties of biomaterials. Another limitation of unicortical defect models relates to the lack of biomechanical loading, which is especially relevant if the biomaterials tested are being considered for use as structural grafts.

Orthotopic evaluation of osteoproductive agents are typically performed in critical-sized diaphyseal defects or in fracture-healing models (Bostrom et al. 1999). These models simulate clinical applications better than unicortical defect models but have several pitfalls. The first type of models, based on the creation of defects that do not heal without implantation of a graft, is designed to simulate nonunions. However, it fails to mimic the clinical situtation encountered in nonunions, because it is the size of the defect, not a compromised healing environment that prevents healing. These models therefore more suited to reproduce massive bone defects, such as those resulting from excision of a neoplasm. In contrast to critical-sized defects, fracture-healing models rely on fracture or osteotomies that heal regardless of treatment. The goal is then to show that an otherwise normal healing process (as opposed to a compromised healing process) can be augmented and possibly accelerated by the therapy tested. This is often challenging and the biologic and biomechanical variability among different models is such that comparison of results between studies is difficult. Compared with unicortical defect models, fracture-healing and critical-sized defect models are more invasive and technically more difficult to use. One of their main limitations is that only one treatment can be evaluated per animal. Evaluation of several treatments and variations between individuals increase the number of animals required to reach

statistical significance, as compared with a model where several implantation sites can be created in each animal.

6. AIMS OF THE STUDY

The general intent of this work was to develop a sound methodology to evaluate the properties of three types of grafting biomaterials with regard to specific clinical applications and to provide insight into the different grafting stategies to enhance bone formation.

Specific goals were as follows:

Study I:

- To evaluate the biomechanical properties of allograft frozen in saline for one year, in bending and torsion.
- To compare these properties to matched bones stored in dry containers

Study II:

- To test the osteoinductive properties of a bone inducing agent (BIA) in higher vertebrates
- To evaluate the biological properties of the agent in orthotopic sites
- To compare the osteoproductive properties of BIA with negative (empty defect) and positive controls (autogenous cancellous graft)

Study III:

- To develop and evaluate a model simulating the environment encountered in revision hip arthroplasty.
- To study the biological properties of several impacted aggregates of large particles in the same animal (higher vertebrate)

Study IV:

- To determine the *in vivo* dissolution rate of a new, silicate-free, idealized, morselized bioactive glass
- To evaluate the osteoproductive properties of the agent, alone or combined with allograft

Study V:

- To evaluate the effect of idealizing particle size distribution of impacted aggregates on their vascularization and incorporation.
- To evaluate the osteoproductive properties of two mixtures of impacted, idealised, morselized tricalcium phosphate and hydroxyapatite.

7. MATERIALS AND METHODS

7.1. GRAFTING MATERIALS:

7.1.1. Corticocancellous bone (Study I)

a. Specimen collection

A total of eleven pairs of metacarpal bones, ten pairs of metatarsal bones, and seven pairs of ribs were cleanly collected from four healthy large mixed breed adult dogs, euthanized after surgical teaching laboratory experiments. Metacarpal and metatarsal bones were collected to obtain a maximal number of paired long bones and enable better use of donors. Ribs were included as another source of corticocancellous bone. All soft tissue was removed by sharp dissection. Epiphyses and cancellous bone were left intact. High-resolution radiographic views (Faxitron Series Model No.43805-N, Hewlett Packard Corp., McMinnville, OR, USA) using nonscreen film (Kodak X-OMAT TL Film, Eastman Kodak Co., Rochester, NY, USA) were obtained for all specimens.

b. Storage

The bones were individually packaged in transparent polyethylene containers (Whirl-pack bags, 9 x 4 1/2 inches, Nasco, Fort Atkinson, WI., USA) and allotted to two groups. One bone of each pair (randomly assigned) was immersed in a polyethylene bag containing 200 ml of normal saline solution (NSS). The contralateral bone was stored in a polyethylene pack without NSS (Figure 8).

Figure 8: Storage of pairs of ribs, metacarpal and metatarsal bones. *One bone is immersed in 200 ml of normal saline solution, while the contralateral bone is stored in a dry polyethylene pack.*

Each container was labeled and stored in a conventional freezer at a constant temperature -20ºC for one year. All frozen specimens were allowed to thaw within their wrappers at 20 to 22ºC, prior to biomechanical testing.

7.1.2. Bone-inducing agent (Study II)

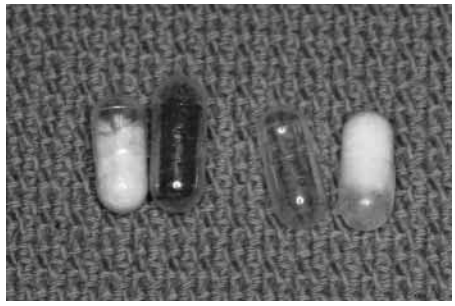
The Saos-2 human osteosarcoma cell line was initially propagated *in vitro* from osteosarcoma tissue of an 11-year-old Caucasian female (Fogh & Trempe 1975). The bone-inducing agent

(BIA) was extracted after resuspension of freeze-dried, acetone-defatted Saos-2 cells in 4M guanidinium hydrochloride (Hunt et al. 1993). The extract was then partially purified by gel filtration chromatography to obtain a 10-50 kDa fraction of BIA. Bioassays were performed in mice prior to implantation in dogs (see Bioassays for osteoinduction).

BIA implants were then prepared for use in dogs by placing 10 mg of BIA and an equal amount of pure bovine collagen (Vitrogen, Collagen Corp., USA) into a #4 gelatin capsule (Eli Lilly and Co., Indianapolis, IN, USA). The implants were gas-sterilized in ethylene oxide and stored in plastic containers, at -70^{0}C, until used. For comparison, control implants were prepared, consisting of 20 mg of pure bovine collagen in a #4 gelatin capsule and a #4 gelatin capsule alone. The last type of implant was fresh autogenous cancellous bone, collected at the time of the operation, and packed into a #4 gelatin capsule (Figure 9).

***Figure 9: Implants used for evaluation of a BIA**, from left to right:*

- *10 mg of BIA and 10 mg of bovine collagen*
- *Fresh autogenous cancellous graft*
- *Empty capsule*
- *20 mg of bovine collagen*



7.1.3. Morselized corticocancellous allograft (Studies III, IV, and V)

Corticocancellous bone was aseptically collected from the femoral and humeral heads as well as distal femora of two adult sheep euthanized as part of an experiment unrelated to orthopedics. Bones were morsellised under aseptic conditions with a bone mill (Aesculap), using the 3- or 6-mm grater (Figure 10).

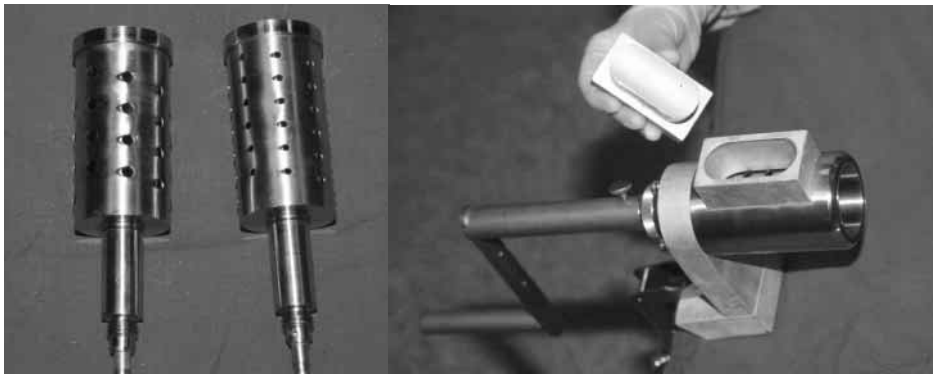
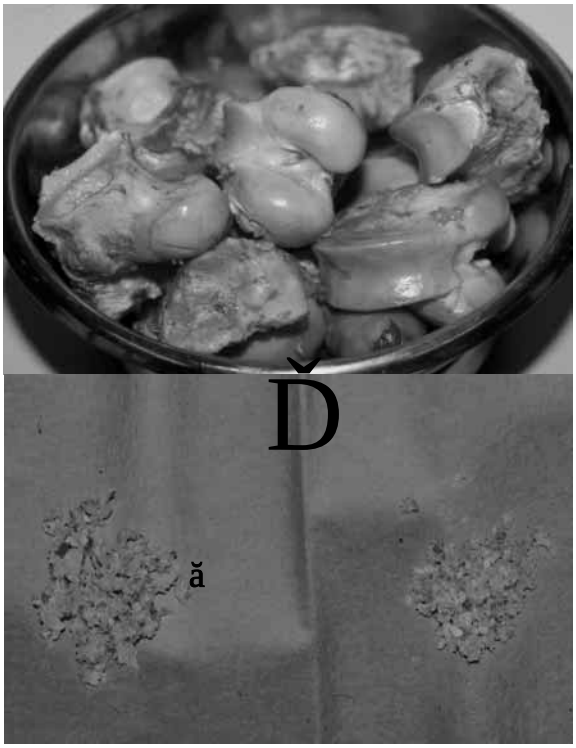


***Figure 10: Aesculap bone mills** - Left: 6-mm and 3-mm graters. Right: mounted bone mill.*

A well-graded specimen of allograft (AlloG) was prepared by mixing 2/3 of small and 1/3 of large particles (Figure 11) by weight.

Figure 11: Preparation of an idealized mixture of morselized allograft. *Distal femora, femoral and humeral heads (Top) were collected aseptically and morselzsed into large (small arrow) and small particles (Bottom right)*



The bone was stored in sterile double-layer polyethylene wrappings at a temperature of -70^0C. A sample of the mixture was submitted for bacterial culture.

7.1.4. Bioactive glass (Studies III and IV)

Corglaes® (Giltech Ltd., Ayr, UK) is a family of bioactive glasses containing pyrophosphate (P_2O_5) rather than silica dioxide (SiO_2) as a network former. These glasses comprise 42-49 mole% of P_2O_5, the remainder as 10-40 mole% of CaO and Na_2O. Test material was prepared by premixing and melting phosphates of sodium and calcium at 1100^0C until stable. The melted glass was cast onto steel and annealed to remove stress. The chemical composition of the material was adjusted based on previous studies (Burnie & Guilchrist 1991), to produce a glass wthat as a 4.0-mm-diameter granule, was estimated to dissolve in 12-14 weeks, in a bone defect. The annealed glass was then crushed, fractionated through a series of eight vibrating sieves, and sterilized by irradiation. Glass particles were provided in eight size ranges (Figure 12):

Particle size ranges (mm)	0.3-0.5	0.5-0.71	0.71-1	1-1.4	1.4-2	2-2.8	2.8-4	4-5.6
Corresponding sieve size (μ)	300	500	710	1000	1400	2000	2800	4000

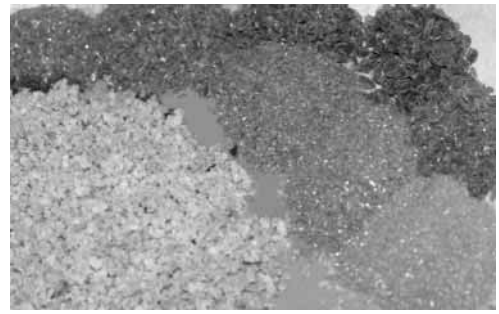


Figure 12: Particles of bioactive glass *were provided in eight size ranges so that an idealized mixture could be prepared by mixing equal weights of each size range. Corticocancellous bone was morselized with a 3- and a 6-mm grater to produce small and large particles.*

Because particles were somewhat irregular in shape, the modified logarithmic line rather than the original Fuller's curve was used to idealize the mixture (Brewster et al. 1999). Based on this curve, logarithmic particle size ranges were determined such that an idealized aggregate of Corglaes® could be prepared by mixing equal amounts (in weight) of each particle size range.
Two groups contained allograft and Corglaes®:
The first consisted of allograft idealized with bioactive glass particles. The particle size distribution of allograft morselized with the 6-mm diameter bone mill used in our study has previously been described (Brewster et al. 1999). Based on this data, the amount of bioactive glass to add to each sieve size was calculated to equalize the weight of the mixture in each particle size range. The particle size distribution of the final specimen therefore matched the theoretical optimum. This mixture of bone idealized with Corglaes® contained 20-25% by volume of bioactive glass particles.
The second consisted of 50% (by volume) idealized, morselized allograft and 50% idealized bioactive glass.

Phase I therefore included six treatment groups:

- Empty defect (negative control)
- Fresh autogenous corticocancellous bone (positive control)
- Idealized corticocancellous allograft (clinical control)
- BG 1: Allograft idealized with particles of Corglaes®
- BG 2: 50/50 mixture of idealized allograft/idealized Corglaes®
- BG 3: Idealized Corglaes®

Anaerobic and aerobic cultures were obtained for all implants prior to storage at -70^0C.

7.1.5. Tricalcium phosphate – hydroxyapatite (Studies III and V)

Calcium phosphate ceramic granules were sterilized by γ-irradiation (BoneSave™; Stryker-Howmedica). The pore size was 400-600 μm, with an interconnectivity pore size of <100 μm and

a porosity of 50%. Two types of granules were used, differing by ratio of tricalcium phosphate (TCP) to hydroxyapatite (HA). The first granules, containing 80% TCP and 20% HA, were serially sieved to obtain a range of eight particle sizes (mm):

0.3–0.5	0.5-0.7	0.7-1.0	1.0-1.4	1.4–2.0	2.0-2.8	2.8–4.0	4.0-5.6

The particle size increments increased logarithmically. An equal weight of each size band produced an idealized stock mixture of 80%TCP-20%HA particles. The second type of granules was treated in an identical manner to produce an idealized stock mixture of 20%TCP-80%HA particles (Figure 13).

A fourth stock mixture was manufactured from 20%TCP-80%HA particles of three size bands only (< 0.5 mm, 1.0-2.0 mm and 2.0-4.0 mm) in a 1:2:1 ratio by weight. This mixture was only approximately idealized (Figure 13).

***Figure 13: Preparation of 4 stock mixtures**, from top to bottom:*

- *Particles of TCP 80-HA 20 in 8 size ranges (BoneSave™)*
- *Particles of HA80-TCP20 in 8 size ranges*
- *Particles of HA80-TCP20 in 3 sizes to approximate idealization*
- *Idealized corticocancellous allograft*

Using the four stock mixtures, four treatments were manufactured for implantation into bone defects in Phase II of the study:

- The positive control consisted of morselized, idealized corticocancellous allograft alone.
- A second group included equal volumes of idealized “TCP80-HA20” particles and idealized allograft.
- Equal volumes of idealized “HA80-TCP20” particles and idealised allograft were mixed to form the third treatment group.
- The last group consisted of idealized corticocancellous bone mixed with an equal volume of 80%HA-20%TCP particles of three sizes only, simulating a clinically expedient method of graft preparation.

7.2. *IN VITRO* STUDIES: BIOMECHANICAL TESTS (Study I)

7.2.1. Four-point bending to failure

Seven pairs of ribs, 5 pairs of metacarpal bones, and 5 pairs of metatarsal bones were loaded in four-point bending to failure. Metacarpal and metatarsal bones were placed between four supports, with the plantar aspect of the bone facing upward (Figure 14). Ribs were placed with the external side facing upward. A bending moment was applied at a rate of 0.5 mm/s, using a modified servohydraulic test frame (MTS Systems Corp., Minneapolis, MN, USA) and a controller (Interlaken Inc., Minneapolis, MN, USA).

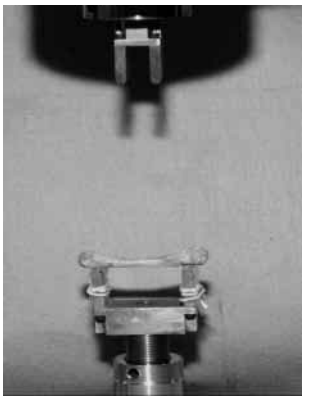


Figure 14: Modified servohydraulic frame used for four-point bending tests.

The distance between one loading point and the corresponding support (d) was used to calculate the bending moment (M, Newton.meter [N.m]):

$$M\ (N.m) = \frac{F\ (N)}{2} \times d\ (m) = 0.0065\ F,$$

where F= Force applied (Newtons)

7.2.2. Torsion tests:

Five pairs of metatarsal and 6 pairs of metacarpal bones were tested to failure in torsion (Figure 15). Ribs could not be mounted on the torque frame because of their shape, and thus, were not tested in torsion.

Figure 15: Testing in torsion to failure.

A 1.1-mm-diameter Kirschner wire was placed through the distal end of each bone before mounting in Wood's metal (Cerro-Bend alloy, Satterlee Co., Minneapolis, MN, USA), to prevent slippage between the bone and the fixture during testing. The bones were potted in Wood's metal heated above its melting point of 70ºC (Rand 1981). The specimens were potted so that the distance of bone exposed between the supports was constant and equal to 35 mm. Specimens were then placed in fixtures attached directly to the servohydraulic torque frame (MTS Systems Corp., Minneapolis, MN, USA). The proximal end of each bone was in the upper fixture, and the distal end in the bottom fixture. Bones were tested to failure in torsion at a rate of 30 degrees/s.

7.2.3. Data analysis:

Bending and torsion forces were directly sampled using an analog to digital converter. Load-deformation curves were analyzed using a scientific graph system (Sigmaplot Software, Jandel Scientific Graph System, Corte Madera, CA, USA). The failure point was assumed to be the point of maximal torque for torsion testing, and the point of maximal bending moment for bending tests. The yield point was the intersection point between the load-displacement curve and a straight line parallel to and 0.1 mm off the regression line of the load-displacement curve.

7.3. *IN VIVO* STUDIES: ANIMAL MODELS (Studies II to V)

7.3.1. Animals and surgical procedures:

a. Bioassays for osteoinduction **(Study II)**

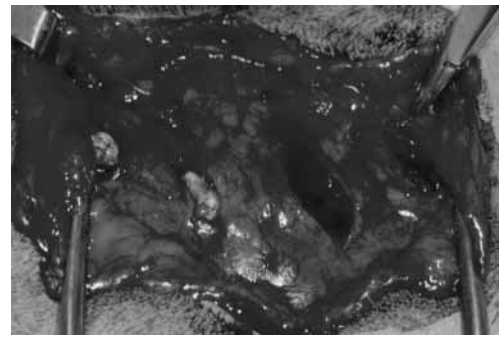
In mice:

To validate the extract prior to use in dogs, 3 mgs of BIA mixed with an equal amount of pure bovine collagen were placed in gelatin capsules and implanted in the Latissimus dorsi muscles of athymic Nu/Nu mice. The sites of implantation were excised two weeks later, fixed for 24-48 hours in 10% phosphate-buffered formalin, and embedded in paraffin. Five-µm-thick sections were stained with haematoxylin and eosin, without decalcification. Sections were then evaluated with a Zeiss EM 10-A electron microscope for evidence of bone formation.

In dogs*:*

Five male Beagles with normal thoracic radiographs were sedated with intramuscular injections of acepromazine (0.04 mg/kg), oxymorphone (0.1 mg/kg), and atropine (0.04 mg/kg). Anesthesia was induced with intravenous (IV) administration of thiopental (10-15 mg/kg) and maintained with a mixture of halothane and oxygen. Lactated Ringer's solution (10 ml/kg/hr IV) was administered during the anesthetic period. Each dog was placed in left lateral recumbency. The left rear leg and the right thoracic wall were prepared for aseptic surgery. An autogenous cancellous bone graft was harvested from the proximal left tibia in a standard fashion (Johnson 1988). The graft was wrapped in a blood-soaked sponge for use later in the procedure and the incision site closed routinely. A dorsoventral incision was made through the skin and the cutaneous trunci muscle over the 5th intercostal space. One of the four implants described in section 7.1.2. (BIA + collagen, collagen alone, gel capsule alone, or autogenous cancellous graft) was placed in each stab incision (Figure 16).

Figure 16: Osteoinductive bioassay in five dogs.
Four stab incisions were made in the right latissimus dorsi muscle over the 4th, 5th, 6th, and 7th intercostal spaces.



A simple interrupted suture of 4-0 wire was placed in the latissimus dorsi as a marker, 1.0 cm dorsal to each site of implantation. The subcutaneous tissue and skin were closed in a routine fashion.

b. Orthotopic evaluation of a bone-inducing agent in diaphyseal defects (Study II)

Two female and two male adult Beagles were included in this experiment. Radiographic and nuclear scintigraphic evaluations of both femurs were within normal limits. Each dog was anesthetized, as previously described, and placed in right oblique recumbency. The left leg was prepared for aseptic surgery, and an autogenous cancellous bone graft was harvested from the proximal left tibia (Johnson 1988).

A standard lateral approach to the left femur was performed. The periosteum was elevated and four 5.0 mm diameter defects were created in the lateral diaphyseal cortex of the femur with an orthopedic drill and a 5.0 mm drill bit (Figure 17). Tape was placed around the drill bit 5 mm from the tip to act as a drill-stop and ensure a constant depth for all of the defects.

Figure 17: Orthotopic evaluation in diaphyseal unicortical defects.
A sterile plastic template was used during drilling to ensure uniform spacing of the defects, 5,0 cm apart, along the diaphysis of the femur. Each of the four treatments was assigned to one of the four drill holes, according to a Latin square design, to evaluate each filling material in all positions along the femoral diaphysis.

A wire suture was placed through the periosteum to mark the proximal end of the template. The distance between the wire and the cortical defects was used to locate the healed defects for histological examination. In each femur, one of the cortical defects was filled with autogenous cancellous graft, one with pure bovine collagen, and one filled with 10 mg of BIA mixed with an equal amount of bovine collagen. All of the implants were packed in #4 gelatin capsules to provide retention at the surgical site. The fourth defect was filled with an empty #4 gelatin capsule. The surgical incision was closed in a routine fashion, and craniocaudal and lateral radiographic views of the left femur were immediately obtained.

c. Use of a metaphyseal defect for evaluation of morselized grafts (Studies III-V):
All mixtures (except autogenous cancellous grafts) were maintained at -70^0C in double polyethylene containers until surgery. A specialized miniature impactor (Figure 18) was designed to impact pellets at the same compactive effort as previously reported (equating to the energy used in a standard human impaction grafting, as determined by force plate analysis of impaction grafting on human femora, Brewster et al. 1999).

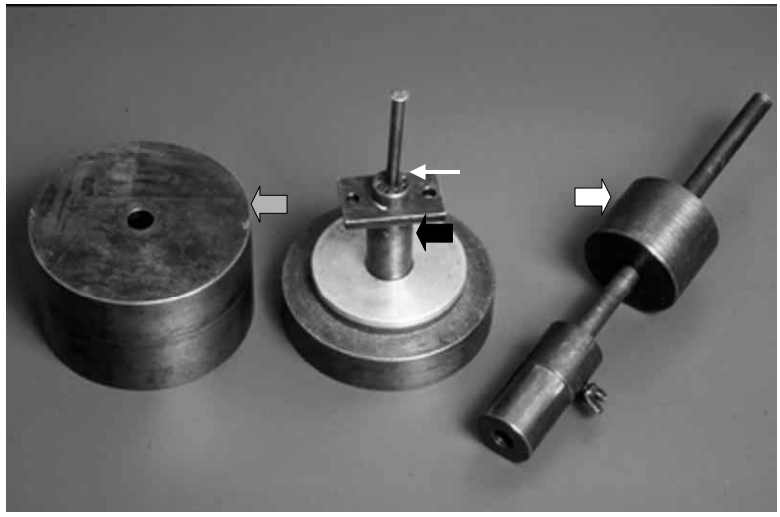


Figure 18: Miniature impactor *- grafting material is placed in a 15-mm-diameter impaction chamber (black arrow). A perforated piston (small white arrow) allows elimination of air and fluid during impaction. The impactor and piston are enclosed within a chamber (left grey block arrow) to improve stability. A brass cylinder (right white arrow) is dropped over the piston, to produce a compaction level similar to that generated during revision hip arthroplasty*

The dimensions of the impactor were adjusted from the original design to produce 15-mm-diameter pellets. The allocation of pellet to defect site was randomized according to a Latin

square design throughout the study, so that each treatment was evaluated more than once in all positions.

Animals:
Twenty-two adult female Grey-face sheep were included in Study IV, to evaluate a silicate-free bioactive glass. This breed of sheep was selected for tits large size, allowing creation of larger bone defects. Body weight varied from 61 to 84 kg, with a mean and standard error of the mean (SEM) of 73 ± 1.04 kg. Evaluation of tricalcium phosphate-hydroxyapatite aggregates in Study V, included eight adult female Grey-face sheep, with body weights ranging from 81 to 91 kg (mean: 84.94 ± 1.17 kg). Data from all animals involved in Studies IV and V were analyzed to evaluate data specifically related to the metaphyseal defect model (Study III). Animals were housed individually from two weeks prior to surgery until the end of the study. Home office regulations for the care and use of laboratory animals were followed throughout the study.

Surgery:
Prior to surgery, animals received intramuscular injections of morphine sulfate (0.1 mg/kg) and penicillin G procaine (10000 IU/kg). Anesthesia was induced with intravenous administration of thiopental (10-14.5 mg/kg) and maintained with a mixture of halothane and oxygen. Both rear legs were clipped and prepared for aseptic surgery. In phase I of the study, a total of six unicortical defects were created in each sheep. Defects were located in the subtrochanteric region of the femur, lateral aspect of the distal femoral metaphysic, and proximal medial tibia in both hind limbs.
The approach to the greater trochanter and subtrochanteric area (Piermattei 1993) was modified to avoid tenotomy of the superficial gluteal muscle and elevation of the insertion of the vastus lateralis. Instead, the subtrochanteric region was exposed by blunt dissection between the fibers of the vastus lateralis muscle (Figure 19a). Exposure was maintained with Gelpi retractors. A teflon plate was designed to guide the insertion of pellets into the defects and to standardize the location of the defects. The plate included two 2.7-mm-diameter holes, 1 cm proximal and distal to a central hole (15 mm in diameter). The proximal femoral defect was created with one edge of the plate aligned with the proximal aspect of the greater trochanter.
A standard approach to the distal femur (Piermattei 1993) was used to expose the lateral aspect of the distal femoral metaphysis. The caudal distal femoral artery was preserved, and the defect created at the level of attachment of the gastrocnemius muscle (Figure 19b).
A 4-cm-long skin incision was made directly over the medial proximal aspect of the tibia. The knee joint was identified by arthrocentesis. A unicortical defect was created at the cranial aspect of the origin of the popliteus muscle, with the edge of the teflon plate positioned just distal to the joint (Figure 19c).

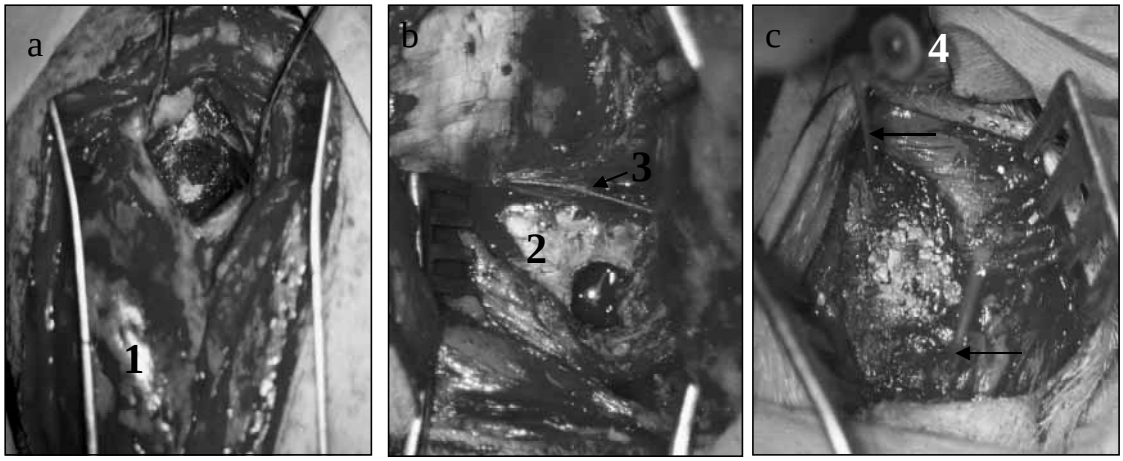


***Figure 19: Surgical approaches**:*
The proximal femoral defect (Figure a) is created after dissection between the fibers of the vastus lateralis muscle (1). The distal femoral defect (Figure b) is located at the level of attachment of the gastrocnemius muscle (2), distal to the caudal distal femoral artery (3). The third defect is drilled in the medial proximal tibia (Figure c), with the edge of the teflon plate positioned just distal to the joint (4).

At each site, a 5-mm-diameter pilot hole was drilled at low speed in the near cortex. A drill-stop was placed 15 mm from the tip of the drill to ensure constant depth of defects. A 15-mm drill was used to enlarge the unicortical defect. Cancellous bone retrieved during creation of the defects was kept in blood-soaked sponges until compaction. Two 2.7-mm cortical screws were inserted into the corresponding holes of the teflon plate to maintain its alignment with the defect during insertion of pellets. The impaction chamber containing the pellet was aligned with the teflon plate over the corresponding bone defect. A mallet was used to advance the piston within the compaction chamber by 15 mm, pushing the pellet into the defect. All defects were therefore filled with pellets measuring 15 mm in diameter and length (Figure 20). The plate and screws were removed and polymethylmethacrylate (Surgical Simplex®P, Howmedica Int. Inc., Ireland) was poured over the defect and into empty screw holes.

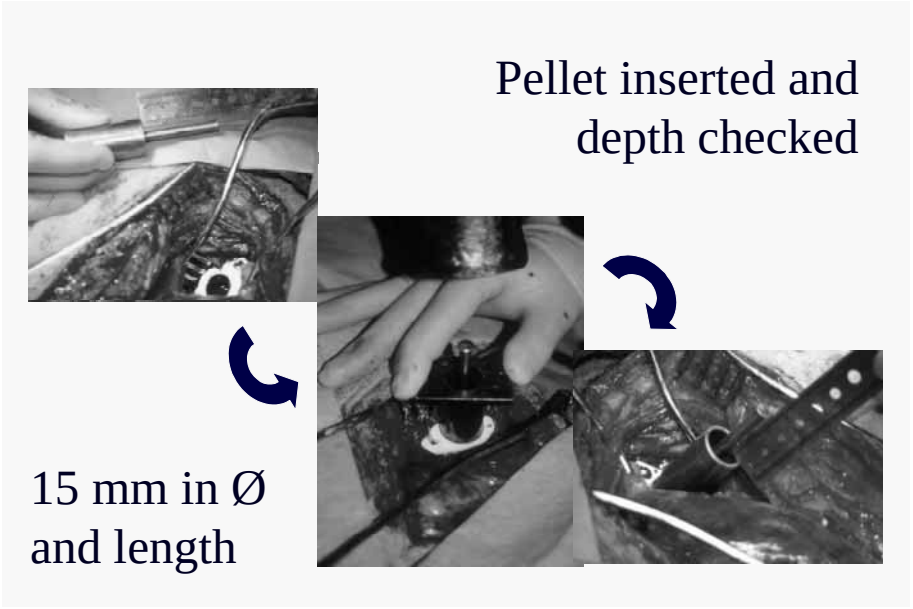


Figure 20: Insertion of pellets within metaphyseal defects.
All defects are filled with pellets measuring 15 mm in diameter and length.

In Study V, eight Grey-face sheep underwent surgical implantation of four defects located in the right and left distal femurs and proximal tibias. The only difference compared with the surgical technique used in Phase I involved the use of two 1.6-mm-diameter Kirshner wires instead of screws to maintain the teflon plate during insertion of pellets (Figure 19c). After removal of the plate, the wires were cut short, bent, and embedded in polymethylmethacrylate to seal each defect.

7.3.2. Postoperative evaluations

a. Osteoinductive bioassays of BIA in dogs (Study II)
The animals were evaluated daily for signs of infection. Thoracic radiographs were obtained immediately after the operation and every two weeks until euthanasia six weeks after implantation. The right latissimus dorsi muscle of each dog was then harvested and grossly examined. High-resolution radiographs (Faxitron Series Model #43805-N, Hewlett Packard Corp., McMinnville, OR, USA), using nonscreen film (Kodak X-OMAT TL Film, Eastman Kodak Co., Rochester, NY, USA), were obtained and three sections were cut over each implantation site. Each section was processed and evaluated histologically.

b. Osteoproductive properties of BIA after orthotopic implantation (Study II):

The dogs were observed daily after the operation for signs of lameness or infection. Nuclear scintigraphy and radiography (lateral and craniocaudal views) of both rear legs were performed at two-week intervals, starting 10 days after the operation. Craniocaudal and lateral nuclear scans were obtained 5 minutes (soft tissue phase), 2 hours (bone phase), and 24 hours (delayed bone phase) after intravenous injection of 1 mCi/Kg of 99 m Technetium-methylene diphosphonate. A 50-pixel region of interest was centered over each of the 4 areas of increased activity in the left femur and corresponding areas of the right femur. A total radioactive uptake of each defect in the left femur was expressed as a percentage of uptake of the corresponding area of the right femur. The change in nucleotide uptake within each defect 10, 24, 38, and 52 days after surgery was calculated for the soft tissue, bone, and delayed (24 hours) bone phases at each evaluation time (Figure 21). The change in nucleotide uptake 10, 24, 38, and 52 days after surgery is calculated for each of the soft tissue, bone, and delayed bone phases, as follows:

$$\frac{\text{Ratio (Left/Right) after surgery} - \text{Ratio (Left/Right) before surgery}}{\text{Ratio (Left/Right) before surgery}}$$

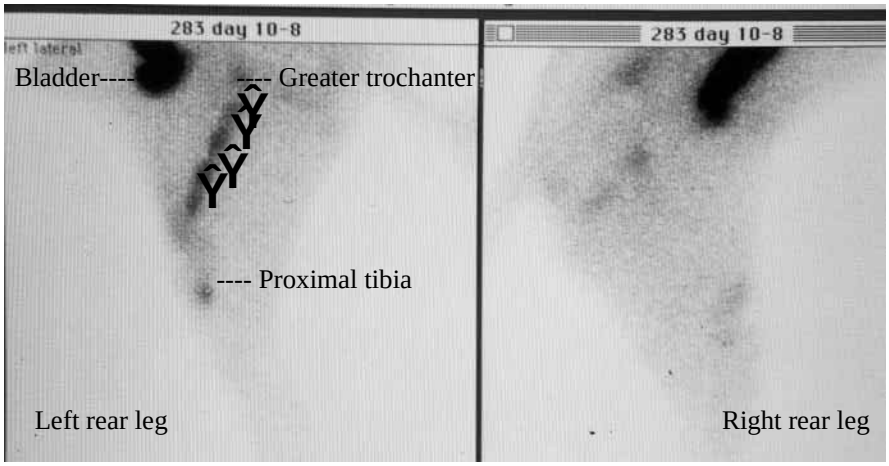


Figure 21: Nuclear scintigraphy, soft tissue phase of dog 283 at day 10.
Areas of increased uptake correspond to the surgical defects (arrows) created in the left femur. The uptake over corresponding areas of the right femur is measured as a control.

All dogs were euthanized by administration of barbiturate overdose eight weeks after the operation. Both femora were harvested, dissected free of soft tissues, except for the periosteum, and examined grossly. Each specimen was fixed in 10% phosphate-buffered formalin. High-resolution radiographs (Faxitron Series Model #43805-N, Hewlett Packard Corp., McMinnville, OR, USA), using nonscreen film (Kodak X-OMAT TL Film, Eastman Kodak Co., Rochester, NY, USA), were obtained for all specimens. With a slow-speed saw (Isomet Plus, Buehler Ltd.,

Lake Bluff, IL, USA), five-mm thick transverse slices were made over the four grafted sites of left femora (Figure 22).

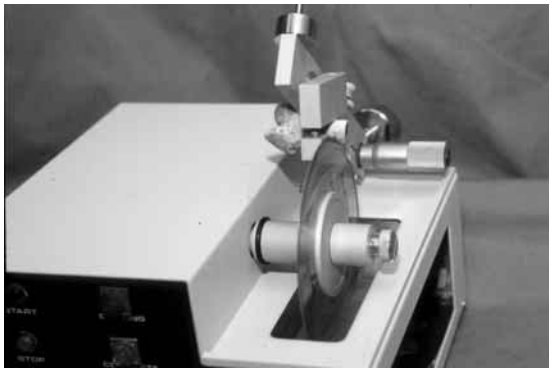


Figure 22: A slow-speed saw *was used to cut 5-mm-thick transverse slices over the four grafted sites of left femora. Slices were also obtained from the corresponding areas of right femora.*

Dual energy X-ray absorptiometry (DEXA) was performed on each slice with a high resolution, single beam, small animal scan (QDR-2000, Hologic, Inc., Waltham, MA, USA). Individualized regions of interest (R1 on figure 23) were constructed over the surgical site of each slice of the left femur. Corresponding regions were constructed on each slice of the right femur using the "compare" feature of the software (Figure 23).

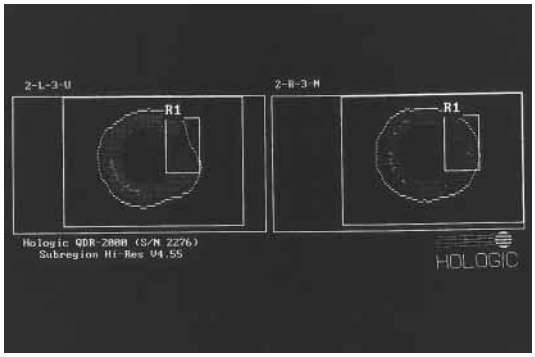


Figure 23: Dual energy X-ray analysis
The bone scans were analyzed for bone mineral content (BMC) and density (BMD).

The ratio:

$$\frac{\text{BMD (or BMC) of a given region of the left femur}}{\text{BMD (or BMC) of the corresponding area of the right femur}}$$

was calculated for each region of interest (R1 on Figure 23).

Two 5 μm-thick sections were cut in the center of each slice on the left femur and stained with hematoxylin and eosin for routine histology. The healing of each defect was evaluated in a blind fashion and graded (Figure 24).

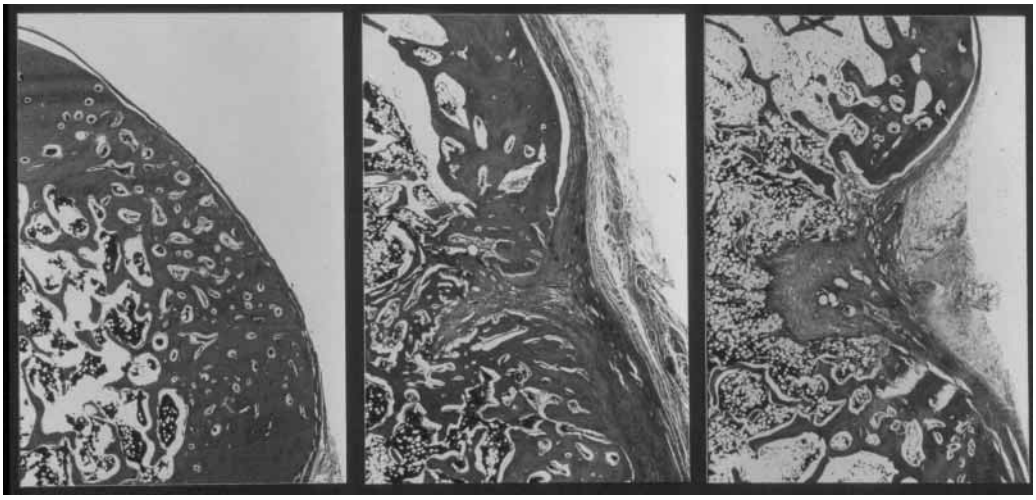
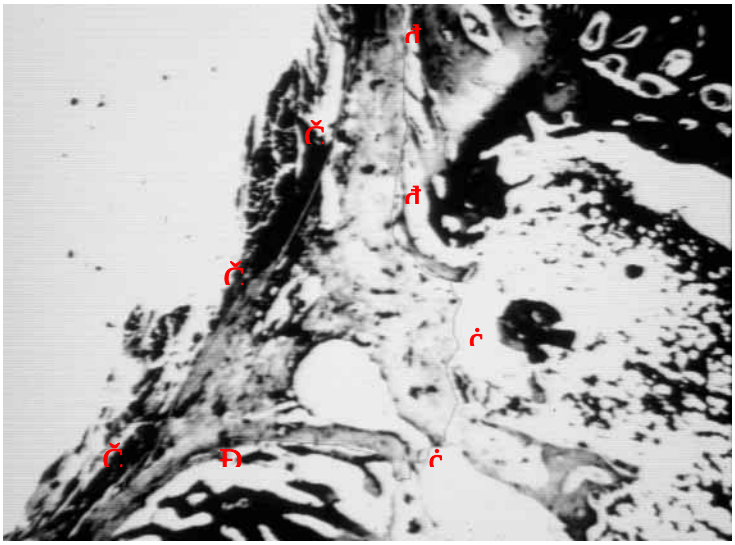


Figure 24: Histological grading system*, from left to right:*

- *Grade 3: defect healed and entirely filled with bone*
- *Grade 2: defect filled with bone and a small amount of connective tissue in the center of the defect*
- *Grade 1: defect filled with connective tissue and a small amount of woven bone along the edges*

Histomorphometric analysis (BIOQUANT-OS/2, R&M Biometrics, Inc., Nashville, TN, USA) of the sections was also performed (Figure 25).

Figure 25: Histomorphometry
The areas of the defect filled with connective tissue (outlined in red) or bone were measured and expressed as a percentage of the total defect area (measured for each defect).



c. Evaluation of idealized aggregates in ovine metaphyseal defects (Studies III, IV, V):
In both Study IV (evaluation of a bioactive glass) and Study V (evaluation of tricalcium phosphate-hydroxyapatite composites), postoperative radiographs were obtained prior to recovery. Animals recovered in a support sling for 12 hours and were then allowed to mobilise over 7 weeks (Phase I, n=11) and 14 weeks (Phase I, n=11; Phase II, n= 8) in individual pens. Analgesia was provided via intramuscular administration of morphine (0.1 mg/kg) 4 hours after surgery and flunixin meglumine (1 mg/kg once daily) for 3 days after surgery. Double pulse oxytetracycline (30 mg/kg at days 23 and 35) and alizarin complexone (30 mg/kg, day 84) were administered at 4 and 12 weeks, respectively. After sheep were euthanized, the distal aorta was perfused with 10% buffered formaldehyde.

Computed tomography:
After removal of the skin, the limbs were fixed in 10% buffered formaldehyde and evaluated with helical computed tomography (CT) (Hi speed advantage, GE medical systems, Milwaukee, WI, USA). Transverse scans of the limbs were made, using 3-mm-thick contiguous slices at 100 kV and 80 mA. The CT scans were evaluated for any orthopedic pathology and the position of the polymethylmethacrylate seal in relation to the corresponding defect was noted. The average pixel value of a 5-mm^2 area (indicated as a square) centered over each defect was divided by that of the intact adjacent cortex (indicated as X) and expressed as a percentage (Figure 26).

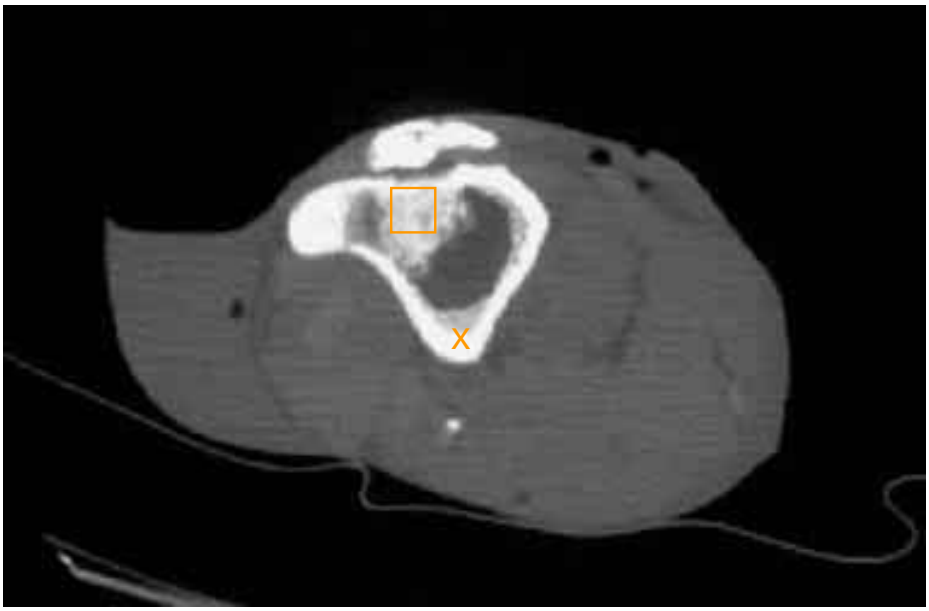


Figure 26: Computed tomography

$$\text{Ratio (\%):} \quad \frac{\text{Average pixel value of the center of the defect (5 mm}^2\text{ square)}}{\text{Pixel value of intact adjacent cortex(X)}} \times 100$$

Histology

All soft tissue was removed from the bones. High-resolution radiographs were obtained prior to cutting 2-cm-wide sections centered over each defect. The sections were divided into two 1-cm-wide blocks. For each defect, one block was decalcified in ethylenediaminetetraacetic acid (EDTA) and embedded in paraffin (Figure 27). At least two 5-µm-thick sections were cut from each block and stained with hematoxylin and eosin. Cortical healing of each defect was evaluated with the same grading system used in the evaluation of a bone-inducing agent (described above). Two independent investigators unaware of the material filling each defect reviewed all sections.

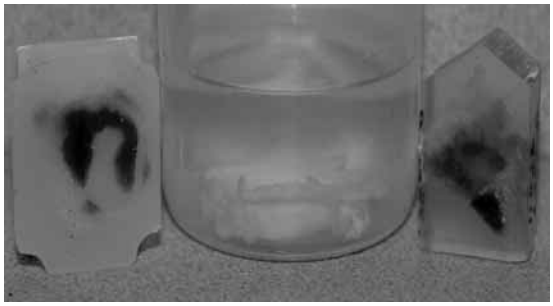


Figure 27: Preparation of sections. *One half of the 2- cm-wide block centered over each defect is decalcified and processed for routine histology (left). The other half is prepared for undecalcified sections (right), histomorphometry and measurement of mineral* apposition rates.

Histomorphometry and fluorescence:

The other 1-cm-wide block of bone from each defect was stored in alcohol, defatted in xylene and embedded in methylmethacrylate (Figure 27). At least three 50-µm-thick sections were cut with a diamond saw (Isomet 2000, Buehler Kraut Kramer, Coventry, UK). In both phases, the mineral apposition rate (MAR) of an area of intact cortex adjacent to the defect was measured. In Study IV, mineral apposition rates were determined at the superficial aspect of the periphery of each defect. In Phase V, mineral apposition rates were measured in four areas within each defect: superficial center, superficial periphery, deep center, and deep periphery. All measurements within the defect were averaged for comparison between treatment groups, side, and location of defect.

At least two sections per block were stained with Paragon and used for histomorphometry. Each entire cross section was captured using a digital camera (3CDD, JVC). Defined areas were measured with image analysis QS 300 (Imaging Associates 1999, version 3.0). The areas within the cortical defect filled with newly formed bone, implants, and fibrous tissue were expressed as percentages of the total area of the cortical defect. The percentage of the defect filled with bone (remnants of allograft and new bone) was also determined.

7.4. STATISTICAL ANALYSES

7.4.1. Biomechanical properties of frozen corticocancellous bone:

Student's paired t-tests were used to identify statistical differences within pairs of bones for each parameter. Six physical properties were evaluated for each testing. Maximal torque (ultimate strength), angular deformation at failure (ultimate strain), torque and angular deformation at yield point, energy absorbed to failure (toughness), and torsional stiffness were analyzed for statistical significance. Maximal bending moment, displacement at failure, bending moment and displacement at yield point, energy absorbed to failure, and bending stiffness were evaluated for bending tests. Data obtained on ribs were analyzed separately from those obtained on metatarsal and metacarpal bones. All differences were considered significant at a probability level of 95% ($p<0.05$).

7.4.2. Evaluation of a bone-inducing agent:

Densitometric and histomorphometric data were analyzed for statistical significance with one-way analysis of variance (ANOVA). Significant differences between treatments were determined with a pairwise comparison of treatment least squares means. Significance level was set at $p < 0.05$.

7.4.3. Evaluation of the metaphyseal defect model and osteoconductive aggregates:

Body weights were compared between groups with a Student's t-test. This test was also used to compare computed tomographic and histomorphometric data between sides of implantation (left and right) and defect location in Phase II. Computed tomographic and histomorphometric data were compared between treatment groups as well as defect location in Phase I (3 sites) using repeated measures analysis of variance (ANOVA). When a significant difference was found, Tukey's "honestly significant difference" (HSD) method was used. Our priority was to compare treatments with the clinical control (allograft) in Phase I; Dunnett's method was used to allow for multiple comparisons. In Phase II, all pairwise comparisons were made for treatment means using Tukey's HSD method.

Nonparametric data were analyzed with the Wilcoxon signed-rank test and Friedman's method. Histological grades consisted of three ordered categories in Phase I. In Phase II, interinvestigator consistency, as well as the implicit assumption of an interval scale, was verified. Mean values were taken from the two observers, resulting in 5 ordered categories. The significance level was set at $p=0.05$ in all tests. All results are given as mean values ± standard error of the mean.

8. RESULTS

8.1. BIOMECHANICAL TESTS (Study I):

No abnormality was detected on examination of high-resolution radiographic views obtained for each bone. Load deformation curves in bending and torsion all had a similar appearance, as illustrated in Figure 28.

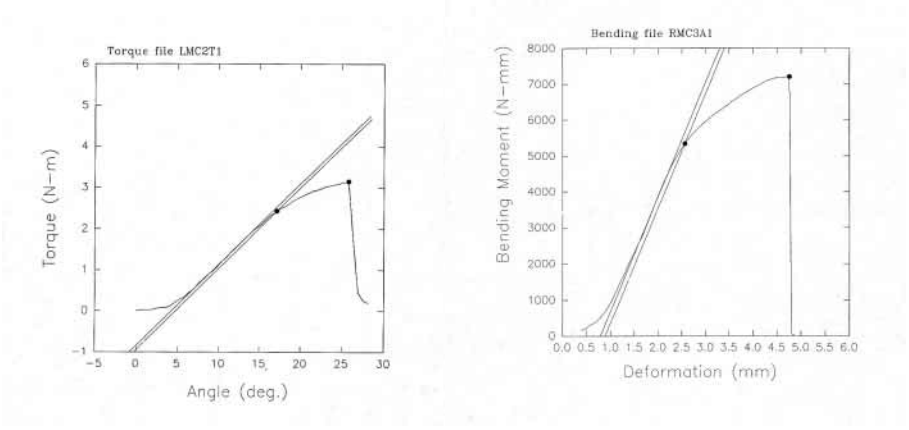


Figure 28: Load deformation curves.
To the left: torsion test of a second left metacarpal bone. Right: bending test of a third right metacarpal bone.

However, differences were found between storage techniques when bones were tested in bending to failure. Results of bending and torsion tests are presented in Tables 6 and 7.

Table 6: Biomechanical properties of ribs and metacarpal (MTC) and metatarsal (MTT) bones, frozen with or without (w/o) normal saline solution (NSS), tested in 4-point bending to failure.

Treatment	No. of bones	Slope (Nm/m)	Displacement at yield point (mm)	Bending moment at yield point (Nm)	Displacement at failure (mm)	Bending moment at failure (Nm)	Energy absorbed at failure (mm Nm^2)
Ribs with NSS	7	815.9± 79.6	1.70± 0.10	1.3± 0.01	5.34± 0.62*	2.08± 0.06	6.98± 0.88*
Ribs w/o NSS	7	904.3± 98.0	1.80± 0.24	1.36± 0.18	4.38± 0.36*	2.09± 0.13	5.44± 0.69*
MTC, MTT with NSS	10	2969.1± 359.7	1.67± 0.12	3.98± 0.39	3.91± 0.36*	5.64± 0.48	13.45± 1.68*
MTC, MTT w/o NSS	10	3259.4± 233.3	1.75± 0.22	4.32± 0.23	3.01± 0.38*	5.78± 0.22	9.31± 1.41*

Data are expressed as mean ± SEM where applicable.
* $p<0.05$

Table 7: Biomechanical properties of metacarpal (MTC) and metatarsal (MTT) bones, frozen with or without (w/o) normal saline solution (NSS), tested to failure in torsion.

Treatment	No. of bones	Slope (Nm/deg)	Angular deformation at yield point (deg)	Torque at yield point (Nm)	Angular deformation at failure (deg)	Torque at failure (Nm)	Energy absorbed at failure (Nm deg)
MTC, MTT with NSS	11	0.17± 0.01	13.70± 0.84	1.99± 0.21	17.43± 0.77	2.34± 0.23	20.62± 2.5
MTC, MTT w/o NSS	11	0.18± 0.01	13.63± 0.49	2.03± 0.14	19.48± 1.47	2.53± 0.18	25.62± 3.0

Data are expressed as mean ± SEM where applicable. Deg=degrees

No significant difference was found in torsion. The displacement and energy absorbed at failure in bending were significantly greater when metacarpal and metatarsal bones were frozen in isotonic saline solution (Figure 29).

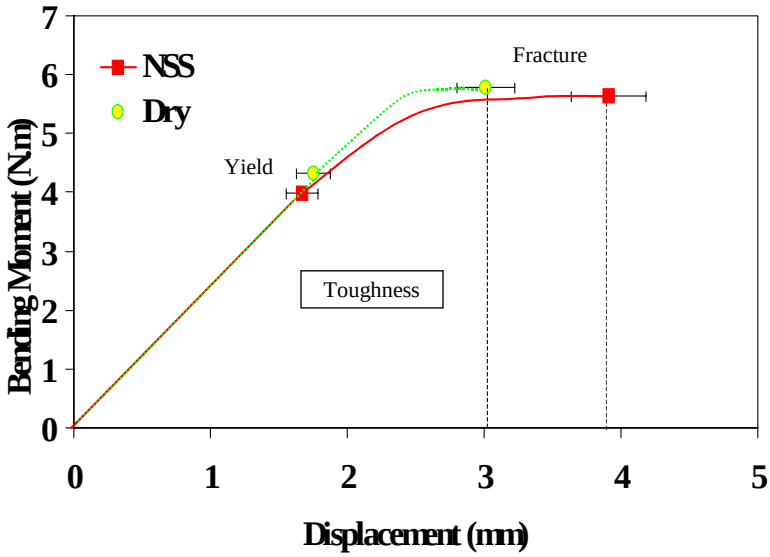


Figure 29: Load deformation curves of metacarpal and metatarsal bones tested to failure in bending *after 1 year of storage at –20°C with or without (Dry) normal saline solution (NSS).*
Graphs are average plots and the points are the averages for each group. Displacement and energy absorbed at failure were significantly greater when bones were stored with NSS.

Similar results were found for ribs (Figure 30):

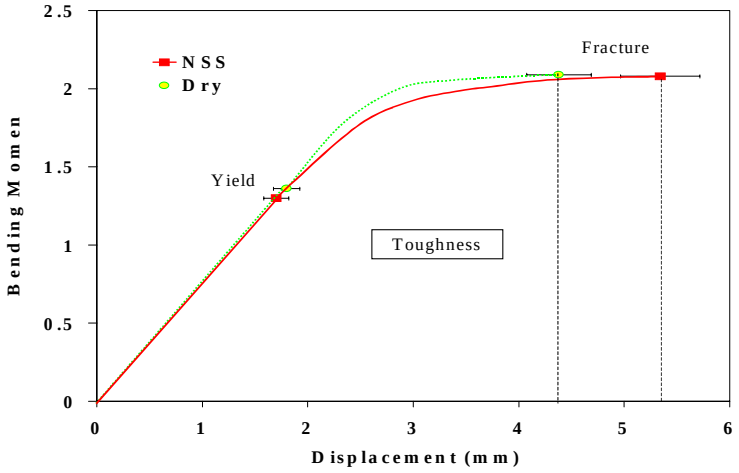


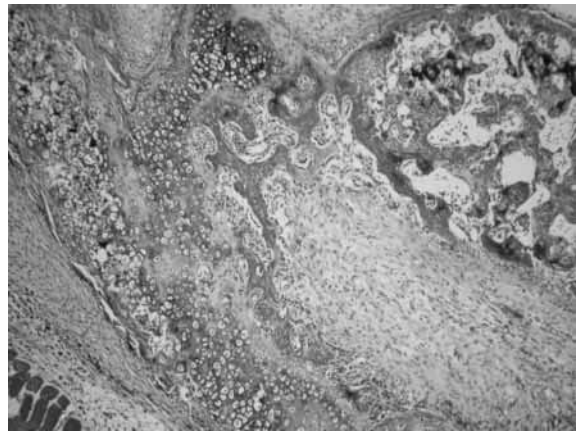
Figure 30: Load deformation curves of ribs tested to failure in bending *after 1 year of storage at –20°C with or without (Dry) normal saline solution (NSS). See Figure 29 for further information..*

8.2. OSTEOPRODUCTIVE PROPERTIES OF A BONE-INDUCING AGENT (Study II)

8.1.1. Ectopic bone formation

An ossicle of bone formed by two weeks after intramuscular implantation of BIA in all mice (Figure 31).

Figure 31: Osteoinductive bioassays in mice . *An ossicle of bone formed within 2 weeks after intramuscular implantation of BIA (hematoxylin and eosin, X2).*



Complications were not encountered during or after the operation in dogs. However, no evidence of cartilage or bone formation was found radiographically, grossly or histologically in any of the intramuscular implantation sites in dogs (Figure 32). A variable degree of fibrosis and condensed muscle cells with large nucleoli, consistent with skeletal muscle regeneration, was found at all implantation sites. Mild to moderate chronic inflammation was seen in half of the surgical sites. Two of the five sites implanted with BIA had increased amounts of chronic inflammation.

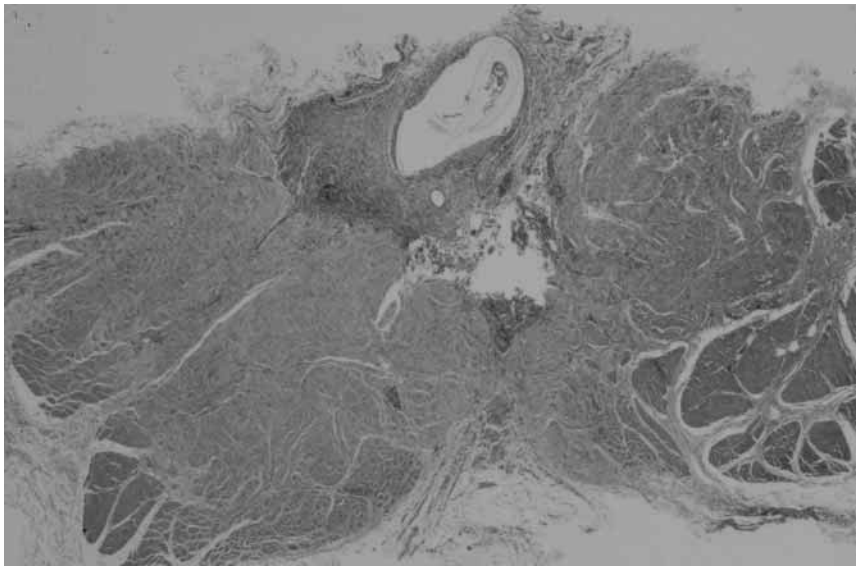


Figure 32: Microphotograph of the latissimus dorsi muscle six weeks after implantation of a bone-inducing agent *(hematoxylin and eosin, X2). Fibrosis and mild inflammation was found, but there was no evidence of cartilage or bone.*

8.2.2. Orthotopic bone formation

The dogs were intermittently weight-bearing lame (grade1) the day after the creation of four cortical defects in the left femora. The lameness resolved within 10 days of surgery.

a. Radiographs

All of the defects were visible on postoperative radiographs and became more radio-opaque over the course of the study (Figure 33). Defects filled with autogenous cancellous grafts were more radio-opaque than other defects immediately after the operation and remained so throughout the study. They were barely visible on radiographs taken 52 days after surgery. Defects filled with BIA implants appeared to be the least radio-opaque of all defects and were easily visualized on radiographs obtained at the end of the study. The radio-opacity of the defects filled with bovine collagen or a gelatin capsule was intermediate. Evidence of microfractures was not seen on high-

resolution radiographs obtained eight weeks after the operation, and all of the defects were readily identified (Figure 33).

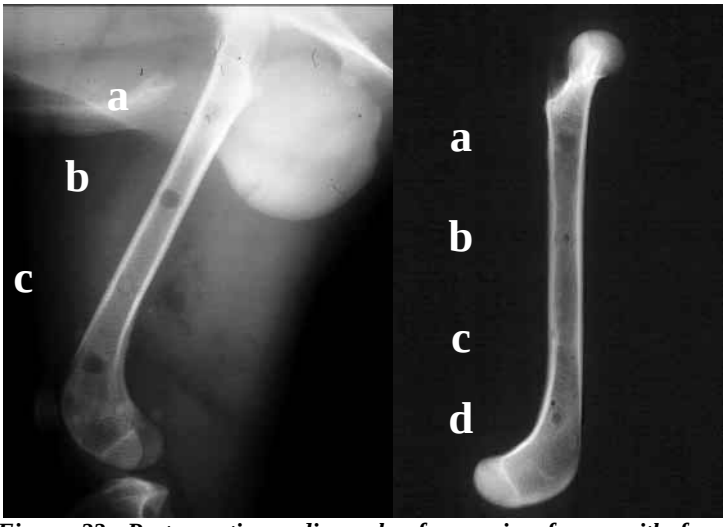


Figure 33: Postoperative radiograph of a canine femur with four cortical defects (Left) and high-resolution radiographs (Right) eight weeks after implantation *of: a) gelatin capsule, b) bovine collagen, c) autogenous cancellous bone, d) 10 mg of BIA mixed with an equal amount of collagen.*

b. Gross examination

Soft tissue reaction was not observed on gross examination of the femora. The defects filled with BIA were grossly more apparent than other defects (Figure 34). They seemed to be filled with a vascular tissue and appeared as dark depressions in the femoral cortex. Defects treated with the other implants varied in appearance. Some looked grossly similar to defects filled with BIA, while other appeared as slight depressions in the femoral cortex.

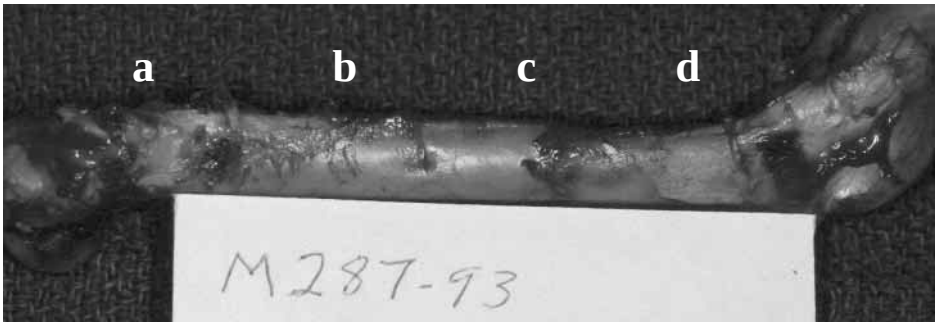


Figure 34: Gross appearance the canine femur radiographed above with four cortical defects, eight weeks after implantation *of: a) gelatin capsule, b) bovine collagen, c) autogenous cancellous bone, d) 10 mg of BIA mixed with an equal amount of collagen.*

c. Nuclear scintigraphy

The results of the nuclear scintigraphic soft tissue phase, bone phase, and delayed bone phase are summarized in Figures 35, 36, and 37, respectively.

The radionucleotide uptake increased in all three phases 10 days after surgery and returned to preoperative values in the subsequent evaluations. In the soft tissue phase (Figure 35), uptake was significantly increased 10 days after surgery in defects treated with autogenous cancellous graft as compared with defects treated with BIA or a gelatin capsule.

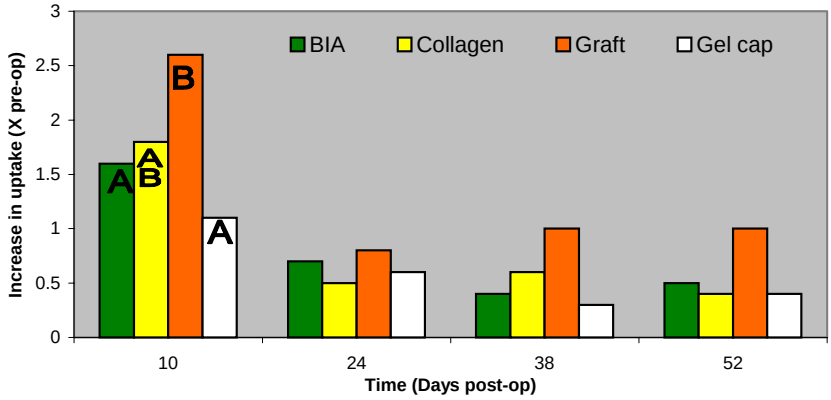


Figure 35: Nuclear scintigraphy – Soft tissue phase *measured 5 minutes after intravenous injection of 99 m Technetium-methylene diphosphonate.*
A,B: groups with different letters differ statistically ($p<0.05$)

There was no difference between groups in the subsequent evaluations. In the bone phase (Figure 36), no significant difference was found in the uptake between treatment groups or between any of the evaluation times.

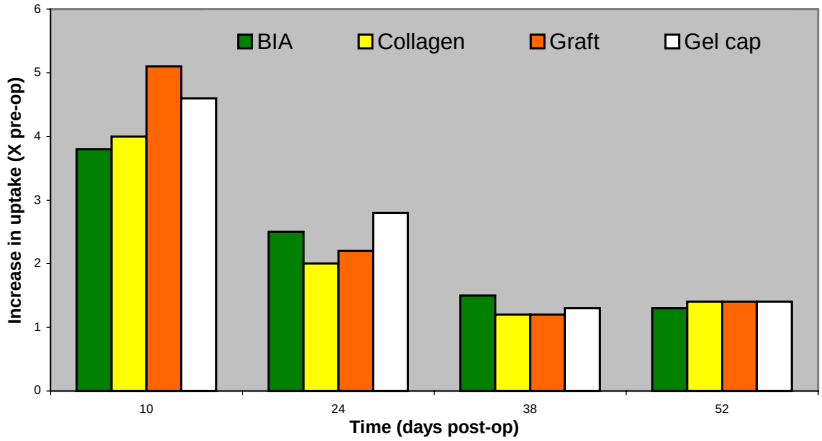


Figure 36: Nuclear scintigraphy – Bone phase *measured 2 hours after intravenous injection of 99 m Technetium-methylene diphosphonate.*

In the delayed bone phase (Figure 37), uptake was significantly increased in defects treated with BIA and autogenous graft as compared with defects treated with collagen or gel capsule at day 10. No significant difference was present between treatment groups at other evaluation times.

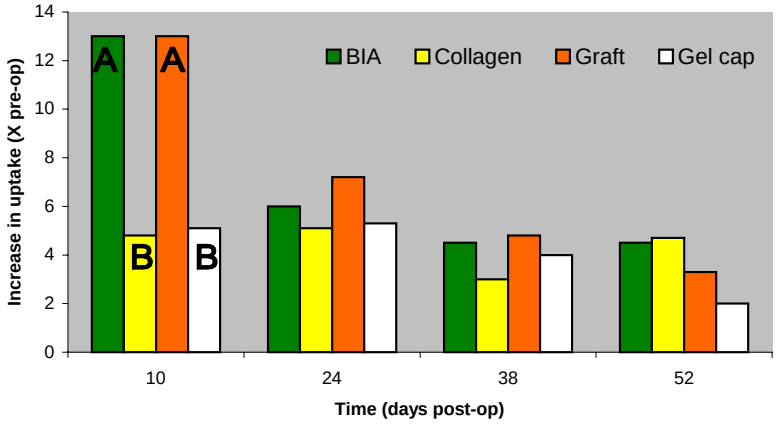


Figure 37: Nuclear scintigraphy – Delayed bone phase *measured 24 hours after intravenous injection of 99 m Technetium-methylene diphosphonate.*
A,B: groups with different letters differ statistically ($p<0.05$)

d. Densitometry

The bone mineral density of the regions containing the defects filled with cancellous graft, BIA, collagen, or a gelatin capsule was 97.0 ± 8.0 (SEM)%, 90.8 ± 10.6%, 87.6 ± 8.3% and 86.0 ± 5.5% that of a corresponding area of the intact femur, respectively. The bone mineral content of the regions containing the defects filled with cancellous bone, BIA, collagen or a gelatin capsule was 93.5 ± 10.6%, 88.8 ± 24.4%, 86.1 ± 16.5%, and 72.0 ± 10.1% that of a corresponding area of the intact femur, respectively. A statistical difference was not found between treatment groups.

e. Histology and histomorphometry

Healing of the cortical defects was evaluated by histology and histomorphometry (Table 8). BIA, collagen, or gelatin residue was not found. The defects treated with autogenous cancellous graft were mostly or entirely filled with trabecular bone. Three of four defects treated with a gel cap alone were healed; however, none of the BIA-filled defects was completely filled with bone eight weeks after the operation. Numerous lymphocytes were found in one of four defects treated with BIA.

Histomorphometry correlated well with the histological grades; defects filled with autogenous cancellous bone contained the greatest amount of bone and least amount of connective tissue of any of the treatment groups. The defects filled with BIA contained the least amount of bone and the greatest amount of connective tissue (Figure 38). Due to the small number of samples, differences were not statistically significant.

Table 8: *Histological grades and histomorphometry of cortical defectsin four dogs eight weeks after treatment with cancellous bone, 10 mg of a bone-inducing agent (BIA) extracted from a human Saos-2 cell line mixed with 10 mg bovine collagen, 20 mg bovine collage, or a gelatin capsule.*

Treatment	Grade I (Nb of defects)	Grade II (Nb of defects)	Grade III (Nb of defects)	% of defect area filled with bone	% of defect area filled with fibrous tissue
Cancellous bone (n=4)	0	1	3	94.2 ± 4.5	5.8 ± 4.5
BIA + Collagen (n=4)	2	2	0	77.2 ± 3.0	22.8 ± 3.0
Collagen (n=4)	0	3	1	87.2 ± 8.6	12.8 ± 8.6
Gelatin capsule (n=4)	1	0	3	89.6 ± 9.1	10.4 ± 9.1

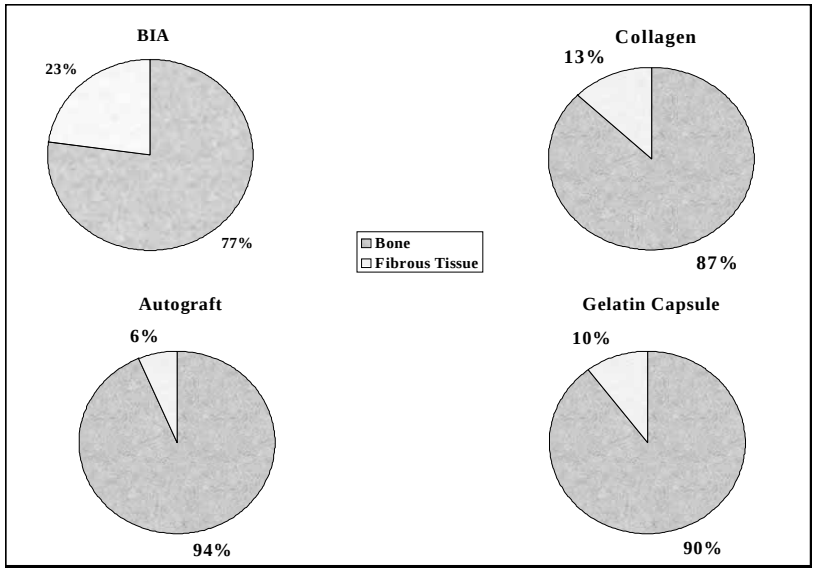


Figure 38: Histomorphometry results*. Areas of the defects filled with bone and fibrous tissue are expressed as % of the total defect area for each treatment group. The defects filled with BIA contained the least amount of bone and the greatest amount of connective tissue.*

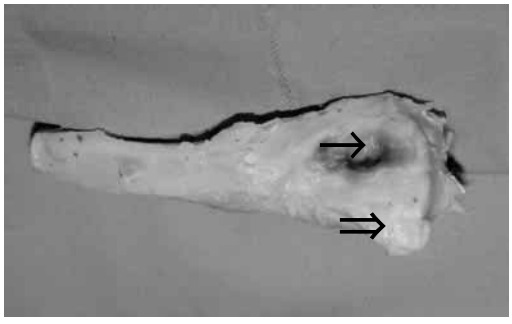
8.3. EVALUATION OF AN OVINE METAPHYSEAL DEFECT MODEL (Study III)

Data obtained on sheep in Studies IV and V was combined to evaluate the ovine metaphyseal defect model.

8.3.1. Complications

Sheep in Study V were heavier than those in Study IV, with a mean body weight of 84.94 ± 1.17 kg (SEM) as compared with 73 ± 1.04 kg. In Study IV, one sheep died of anesthetic complications during recovery. In addition, four of 21 sheep fractured one femur within a week of surgery (19%). The fractures were spiral, originating from the distal femoral defect in three sheep. Two of these sheep had been operated on at the beginning of the study, and the distal defect of both fractured femora was found to be too close to the diaphysis, proximal to the caudal distal femoral artery. Landmarks were subsequently adjusted and defects created distal to the caudal distal femoral artery, at the level of attachment of the gastrocnemius muscle. In the fourth sheep, the fracture originated from the proximal femoral defect. No postoperative complication occurred in study V.
Migration of the cement seal was noted in 25 out of 102 defects (24.2%) in study IV: 18 defects (17.6%) were completely exposed and 7 defects (6.9%) were partially exposed. Polymethylmethacrylate penetrated within 5 of the 17 empty defects (29.4%, Figure 39). These five defects were excluded from the study. All defects in the proximal femur were found to reach the intertrochanteric fossa on postmortem examination. None of these complications occurred in Study V.

Figure 39: Bone cement seal engaging an empty defect in the proximal tibia.
Polymethylmethacrylate has been elevated (block arrow) from the bone to visualize the unhealed defect (arrow). This defect was excluded from the study.



8.3.2. Effect of implantation site

Computed tomography results evaluating the effects of side and site of implantation are summarized in table 9. No difference was found between sites of implantation in study IV, whereas in study V, distal femoral defects were denser than defects located in the proximal tibia.

Table 9: Computed tomography.
The mean pixel value measured in a 5-mm^2 area centered over the defect is expressed as a percentage of the pixel value of intact adjacent cortical bone (% ± standard error). Effect of side (all sites amalgamated) and site (right and left included) of implantation.

	Study IV 7 weeks	Study IV 14 weeks	Study V 14 weeks
Proximal femur	23.13 ± 5.09	23.65 ± 2.98	N/A
Distal Femur	19.71 ± 5.29	27.8 ± 3.05	67.48 ± 3.57*
Tibia	21.05 ± 5.42	18.58 ± 3.05	52.15 ± 3.57*
Left limb	21.66 ± 4.01	24.93 ± 2.47	61.24 ± 3.57
Right limb	21.13 ± 4.61	21.76 ± 2.47	58.39 ± 3.57

** p<0.05: density was higher in distal femoral defects than tibial defects in Study V*

Side and location of the defect seemed to influence the healing of the defects created in Study V; distal femoral defects scored higher than tibial defects (Table 10). Although not statistically significant, a similar trend was found in Study IV.

Table 10: Histological grades of cortical defects filled with morselized impacted grafting materials.
Grades increase from 1 to 3 with improved healing (± standard error). Effects of side (all sites amalgamated) and site (right and left included) of implantation.

	Study IV 7 weeks	Study IV 14 weeks	Study V 14 weeks
Proximal femur	2.17 ± 0.23	2.09 ± 0.17	N/A
Distal femur	1.92 ± 0.24	2.27 ± 0.18	2.44 ± 0.15*
Tibia	1.5 ± 0.23	1.86 ± 0.17	1.81 ± 0.15*
Left limb	1.94 ± .018	2.21 ± 0.14	2.03 ± 0.15
Right limb	1.78 ± 0.2	1.94 ± 0.14	2.22 ± 0.15

** p<0.05: distal femoral defects scored higher than tibial defects in Study V*

Histomorphometry correlated well with histological grades. In Study IV, tibial defects contained less bone and more fibrous tissue than proximal femoral defects 7 weeks after surgery but not at week 14 (Table 11). New bone predominated over dead allograft within defects, representing 89 ± 2.89% and 94.22 ± 6.08% of all bone within the defect area at 7 and 14 weeks respectively. Tibial defects contained less new bone than proximal and distal femoral defects at 7 weeks, but no difference was found at 14 weeks.

Table 11: Histomorphometric analysis in Study IV.
Area of cortical defects filled with bone (newly formed bone and allograft), new bone, and fibrous tissue at, 7 and 14 weeks after surgery (% ± standard error). Effects of side (all sites amalgamated) and site (left and right included) of implantation.

		% bone	% new bone	% fibrous tissue
Proximal Femur	7 weeks	36.28 ± 3.84*	31.65 ± 3.12[A]	64.17 ± 3.78*
	14 weeks	42.68 ± 6.89	40.63 ± 6.55	58.99 ± 6.33
Distal Femur	7 weeks	30.91 ± 4.15	29.54 ± 3.37 [A]	69.25 ± 4.09
	14 weeks	47.22 ± 6.47	45.24 ± 6.15	44.76 ± 5.95
Tibia	7 weeks	18.47 ± 3.07*	15.98 ± 2.49 [B]	81.87 ± 3.02*
	14 weeks	53.83 ± 5.97	49.65 ± 5.67	43.12 ± 5.48
Left limb	7 weeks	32.25 ± 2.82	28.12 ± 2.29	67.68 ± 2.78
	14 weeks	49.19 ± 5.13	45.47 ± 4.88	49.87 ± 4.72
Right limb	7 weeks	24.86 ± 3.07	23.33 ± 2.49	75.85 ± 3.02
	14 weeks	46.63 ± 5.34	44.87 ± 5.07	48.04 ± 4.91

**:p<0.05: Defects in the proximal femur contained more bone and less fibrous tissue than tibial defects at 7 weeks.*
[A,B]: Different letters indicate differences between implantation sites. Defects in the proximal and distal femur contained more new bone than tibial defects at 7 weeks.

In Study V, greater amounts of synthetic agents were found after 14 weeks in the distal femur compared to the proximal tibia (Table 12). A tendency (p=0.2) was also present for distal femoral defects to contain more bone than tibial defects.

Table 12: Histomorphometry in Study V.
Area of the defects filled with bone and with implants 14 weeks after implantation of tricalcium phosphate and hydroxyapatite aggregates (% of defect area ± standard error).

	Phase II (14 weeks) % bone	Phase II (14 weeks) % implants
Distal femur	47.8 ± 2.7	43.02 ± 2.55*
Tibia	42.8 ± 2.79	29.86 ± 2.55*
Left limb	42.84 ± 2.79	36.93 ± 2.55
Right limb	47.77 ± 2.7	35.95 ± 2.55

** p<0.05: Greater amounts of more synthetic agents were found in the distal femur than tibia.*

The bone-labelling regimen selected in this study was adequate for visualization of bone fluorochromes and measurement of mineral apposition rates (Figure 40).

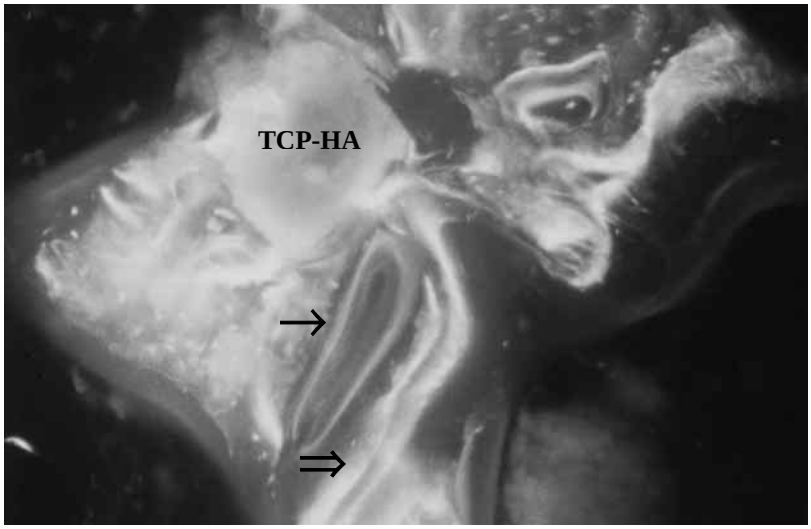


Figure 40: Bone fluorescence in Study V.
The double pulse of oxytetracycline (block arrow) and alizarin complexone (arrow) are identified under polarized light to measure mineral apposition rates at 4 and 12 weeks after surgery, respectively.

Mineral apposition rates did not differ between sites of implantation and treatments throughout the study. The double pulse of oxytetracycline administered at 4 weeks after surgery allowed measurements of mineral apposition rates (MAR) equal to 1.88 ± 0.11 and 2.1 ± 0.07 μm/day in Study IV sheep euthanized at 7 and 14 weeks, respectively. MARs measured in the defects were generally 40-50% higher than those measured in matched adjacent intact cortex during the same

period (1.54 ± 0.19 and 1.57 ± 0.09 μm/day in sheep euthanized at 7 and 14 weeks, respectively). The mineral apposition rates measured in the defects at 12 weeks decreased to 1.13 ± 0.2 μm/day. Similar results were obtained in Study V, where MARs decreased from 2.57 ± 0.3 μm/day at 4 weeks to 1.45 ± 0.18 μm/day at 12 weeks. Within each defect of Study V, MARs at 4 weeks after surgery were higher in the center rather than the periphery of the defect, especially at the most superficial aspect of the cortical defect (Table 13). No difference was found at 12 weeks, but there was a trend (p=0.06) for deeper positions to have lower MARs than superficial positions.

Table 13: Mineral apposition rates *(μm/day ± standard error of mean) measured at the superficial periphery, superficial center, deep periphery, and deep center within 31 metaphyseal defect at 4 and 12 weeks after surgery.*

	4 weeks	12 weeks
Superficial periphery	2.29 ± 0.08*	1.63 ± 0.08
Superficial center	2.87 ± 0.08*	1.39 ± 0.08
Deep periphery	2.41 ± 0.08	1.40 ± 0.08
Deep center	2.54 ± 0.08	1.38 ± 0.08

* $p<0.05$: MAR were higher in the superficial center of defects than at the periphery.

8.3.3. Value of the model in the evaluation of impacted aggregates

The limitations related to the number of defects per limb and the migration of the cement seal are presented under complications. All defects were identified by computed tomography. The placement of Kirshner wires in Study V did not impede measurements of the pixel value within the defect.

Empty defects used as negative controls in Study IV had the lowest density of all groups at 7 weeks, but their density increased thereafter. At 14 weeks, they did not differ from the positive (autogenous graft) and clinical (allograft) controls (Figure 41, Section 8.4.1.). In Study IV, the density of defects filled with compacted allograft or autogenous cancellous graft was similar at 7 and 14 weeks after surgery. No difference was found in any other parameter evaluated between autogenous bone and allograft in Study IV. Allograft seems to act as a suitable positive control for evaluation of synthetic biomaterials with the model described here. It was therefore selected as a control group in Study V.

Healing of the defects was also evaluated via histology and histomorphometry. In both phases, a layer of fibrous tissue was found at the graft-cement junction. In Study IV, particles of dead bone were identified in all defects filled with allograft, either alone or mixed with synthetic agents. Although the presence of autogenous cancellous graft within the defect was more difficult to verify, all defects in this treatment group healed well. Remnants of the synthetic agent tested in Study IV could only be identified in one specimen at 7 weeks. However, remnants of implants could be seen 14 weeks after surgery in all defects filled with the ceramics evaluated in Study V. Migration of pellets therefore seems unlikely.

The results related to individual treatment groups are presented in sections 8.4.and 8.5. The model was useful in evaluating the incorporation, biocompatibility, and i*n vivo* dissolution rates

of the materials tested. Differences were found between treatments groups in both phases, allowing comparison of the osteoconductive properties of the agents tested.

8.4. *IN VIVO* DISSOLUTION OF A SILICATE-FREE BIOACTIVE GLASS (Study IV)

Aerobic and anaerobic cultures of all implants used in this study were negative. Complications are reported in the section dealing with the evaluation of the model.

8.4.1. Computed tomography

Computed tomography results are summarized in Figure 41.

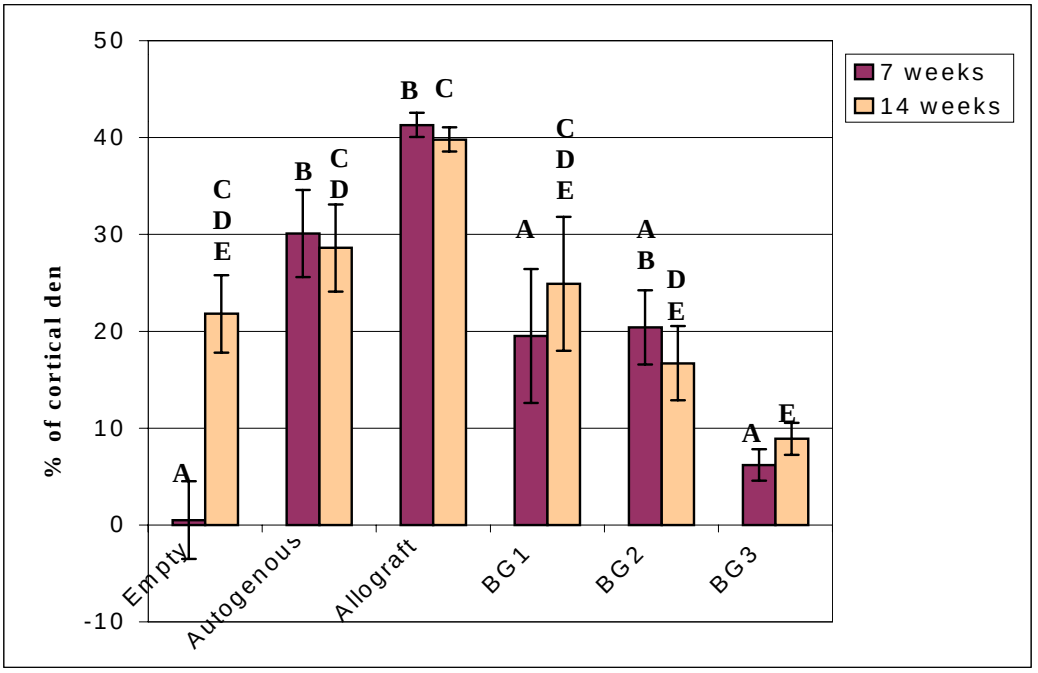


Figure 41: Computed tomography. *The mean pixel value measured in a 5-mm^2 area centered over the defect is expressed as a percentage of the pixel value of intact adjacent cortical bone (% ± standard error).*

- *Empty: empty defect (negative control)*
- *Autogenous: fresh autogenous corticocancellous bone (positive control)*
- *Allograft: corticocancellous allograft (clinical control)*
- *BG1: Allograft idealized with particles of Corglaes®*
- *BG2: 50/50 mixture of idealized allograft/Corglaes®*
- *BG3: Idealized Corglaes®*

Different letters indicate statistical difference between treatments (A,B at 7 weeks, CDE at 14 weeks): Empty defects and treatment BG3 are different from the Allograft and Autograft groups at 7 weeks. At 14 weeks, treatments BG2 and BG3, containing 50% and 100% bioactive glass, respectively, are different from the Allograft group.

The pixel value (mean ± SEM) of intact cortical bone was 1860 ± 46.0 and did not differ between implantation sites. The pixel value (mean ± standard deviation) of pellets prior to implantation was 545.1 ± 210.7 for idealized allograft, 694.5 ± 214.7 for allograft idealized with Corglaes®, 1245.6 ± 217.3 for the 50/50 mixture of allograft/Corglaes®, and 1734.5 ± 204.1 for idealized Corglaes®. Other computed tomography results are presented in Figure 41. Seven weeks after surgery, the density of empty defects and defects filled with idealised Corglaes® was lower than that of defects filled with allograft ($p<0.05$). Fourteen weeks after implantation, defects filled with allograft had a higher pixel value ratio than defects filled with 50% or more Corglaes® (treatment groups 5 and 6). The pixel value of defects filled with allograft idealized with Corglaes® was similar to that of defects filled with autograft or allograft.

8.4.2. Histology

In all defects, a layer of fibrous tissue was found at the cement-graft junction, consistent with a reaction to polymethylmethacrylate. Mild inflammation was found in only three defects in one sheep euthanized at 14 weeks: one of the defects was filled with allograft idealized with Corglaes®, the second with the 50/50 mixture, and the third with allograft. Although particles of dead bone were identified in all defects filled with allograft, remnants of Corglaes ® were found in only one specimen at seven weeks after surgery (Figure 42).

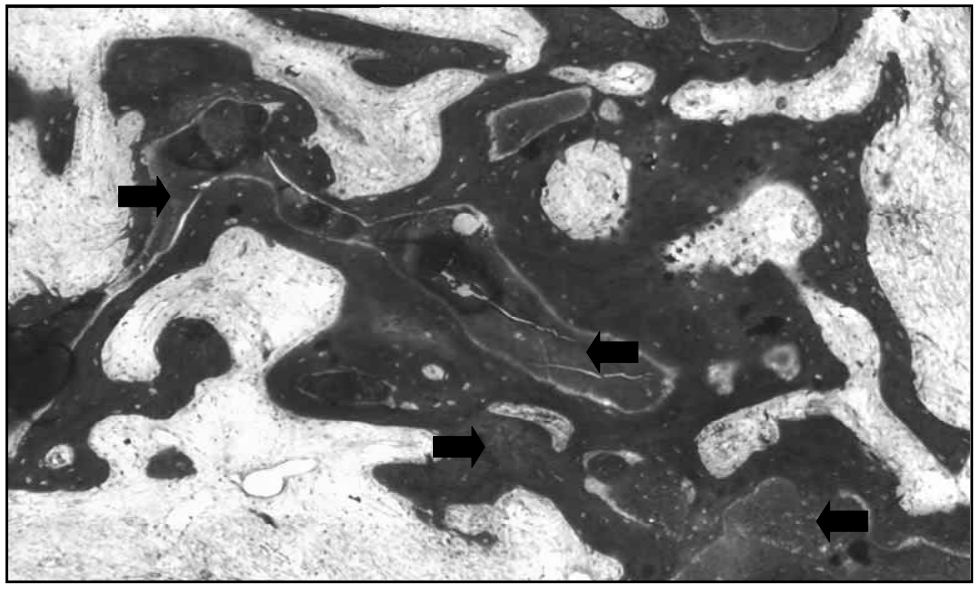


Figure 42: Paragon-stained undecalcified section *(magnification X10) of the trabecular area: remnants of bioactive glass (black arrows) 7 weeks after implantation of an impacted idealized aggregate of Corglaes® in the right distal femoral metaphysis of a sheep. The perimeter of particles attached to new bone varied between 63.18% and 87.69%.*

No particle of Corglaes® was seen within cells, consistent with removal of controlled release glasses by dissolution rather than cellular degradation. The healing of the defect was poor and a histological grade of 1 (out of 3) was assigned to this defect. The area of the cortical defect contained 7.8% bone (all new), 13% bioactive glass, and 78.6% connective tissue.
Histological grades did not differ between treatment groups seven weeks after surgery (Figure 43). However, defects containing more than 50% Corglaes® obtained lower grades than the clinical control at 14 weeks (Figure 43).

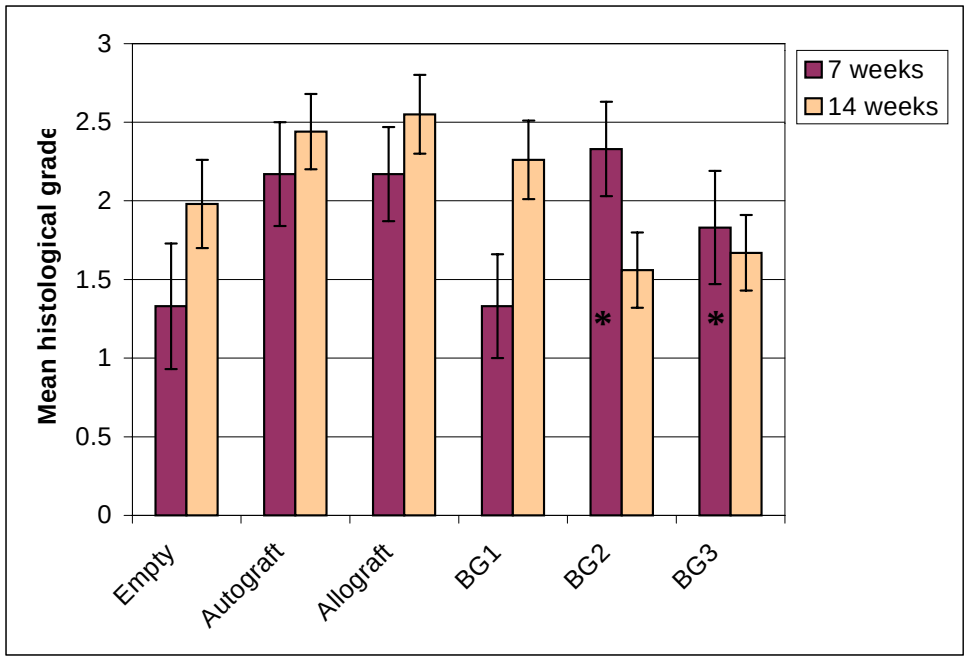


Figure 43: *Histological grades (mean) of metaphyseal defects filled with impacted morselized grafts. Grades increase from 1 to 3 with healing (mean ± standard error).*

- *Empty: empty defect (negative control)*
- *Autogenous: fresh autogenous corticocancellous bone (positive control)*
- *Allograft: corticocancellous allograft (clinical control)*
- *BG1: Allograft idealized with particles of Corglaes®*
- *BG2: 5 50 mixture of idealized allograft / Corglaes®*
- *BG3: Idealized Corglaes®*

* *indicates a statistical difference between the treatment and allograft (clinical control): Treatments BG2 and BG,3 containing 50% and 100% bioactive glass, respectively, are different from the Allograft group.*

8.4.3. Histomorphometry

Histomorphometric data confirmed histology results (Table 14). Most (85.56 ± 7.2%) of the bone found within each defect containing allograft alone or mixed with synthetic graft consisted

of newly formed bone seven weeks after implantation. The rest of the defect was filled with particles of dead bone and fibrous tissue. By 14 weeks, 94.97 ± 4.3% of the bone found in these defects was new, with very little evidence of allograft remnants.

***Table 14*: Histomorphometric analysis -** *percentage of the defect area filled with bone, new bone, and fibrous tissue (mean ± standard error).*

- *Empty: empty defect (negative control)*
- *Autogenous: fresh autogenous corticocancellous bone (positive control)*
- *Allograft: corticocancellous allograft (clinical control)*
- *BG1: Allograft idealized with particles of Corglaes®*
- *BG2: 50/50 mixture of idealized allograft/Corglaes®*
- *BG3: Idealized Corglaes®*

		% bone	% new bone	% fibrous tissue
Empty	7 weeks	11.83 ± 7.19 *	12.90 ± 5.84 *	90.33 ± 7.08 *
Defect	14 weeks	52.68 ± 10.88	50.36 ± 10.35	47.73 ± 10.93
Autogenous	7 weeks	38.90 ± 4.55	35.03 ± 3.69	61.07 ± 4.48
Graft	14 weeks	52.39 ± 8.88	46.30 ± 8.45	47.82 ± 8.93
Idealised	7 weeks	36.34 ± 5.09	31.62 ± 4.13	63.39 ± 5.01
Allograft	14 weeks	64.88 ± 8.88	58.75 ± 8.45	33.49 ± 8.93
BG1	7 weeks	29.69 ± 4.55	25.31 ± 3.69	70.14 ± 4.48
BG1	14 weeks	49.48 ± 8.88	48.63 ± 8.45	50.83 ± 8.93
BG2	7 weeks	39.60 ± 4.55	35.51 ± 3.69	60.03 ± 4.48
BG2	14 weeks	28.78 ± 8.43 #	28.33 ± 8.02 #	71.27 ± 8.47 #
BG3	7 weeks	14.96 ± 5.87 *	13.97 ± 4.77 *	85.64 ± 5.78 *
BG3	14 weeks	38.78 ± 8.88 #	38.64 ± 8.45	60.82 ± 8.93 #

*For each parameter, * and # indicate a statistical difference between the group and the allograft (clinical control) at 7 and 14 week, respectively.*

Empty defects and those filled with Corglaes® alone contained less new bone and more fibrous tissue than defects filled with allograft seven weeks after surgery. Empty defects gradually filled with bone and by 14 weeks contained as much bone as defects treated with allograft, autograft, or

allograft idealized with Corglaes® ($p>0.05$). However, defects filled with Corglaes® alone or mixed with allograft in a 50/50 ratio contained less bone and more fibrous tissue than the clinical control.
Mineral apposition rates (MAR) did not differ between treatment groups throughout the study.

8.5. OSTEOCONDUCTIVE PROPERTIES OF IMPACTED TRICALCIUM PHOSPHATE – HYDROXYAPATITE

Postoperative radiographs showed that the defects were correctly positioned. No perioperative complications were encountered. All sheep were bearing weight on the affected limbs within five days of surgery. Minor soft tissue inflammation was noted on gross examination of two defects where the polymethylmethacrylate plaque had loosened.

8.5.1. Computed tomography

The results of CT-derived densitometry are summarized in Figure 44. Defects implanted with mixtures containing TCP-HA had higher mean values than defects treated with allograft ($p<0.05$). Mean values for defects treated with "TCP80" (BoneSave™), "HA80", and "HA80-3 sizes" were not significantly different from each other ($p>0.05$).

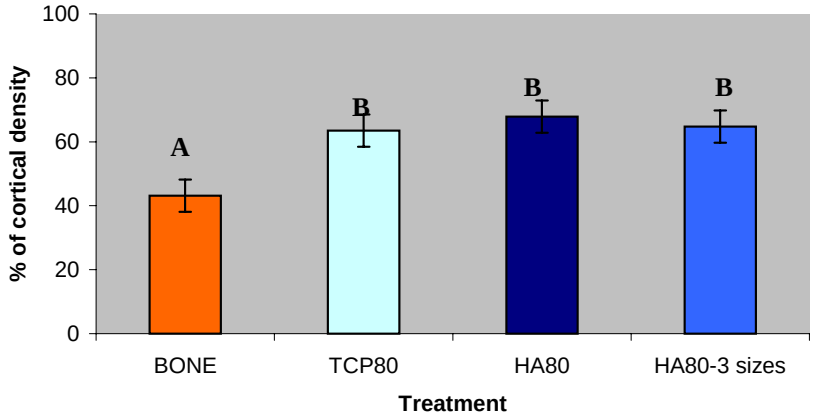


Figure 44: Computed tomography *– values are expressed as a percentage of defect density over adjacent cortical density (means ± SEM).*

- *Bone: impacted idealized morselized allograft*
- *TCP80: idealized mixture containing 80%TCP-20%HA (BoneSave™) in a 50/50 ratio with bone*
- *HA80: idealized mixture containing 80%HA-20%TCP in a 50/50 ratio with bone*
- *HA80-3 sizes: partially idealized mixture containing 80%HA-20%TC in a 50/50 ratio with bone*

A,B: significant differences between groups ($p<0.05$)

8.5.2. Histology

Histological examination of defects implanted with allograft ("Bone") showed that there was little remaining dead bone, with trabecular bone predominating. One defect designated "Bone" was devoid of all treatment material and was excluded from the study. No evidence of inflammatory response was present in this defect so it was assumed that the mixture had become dislodged at some point.

The healing of defects treated with TCP-HA mixtures was less consistent, with variable proportions of trabecular bone, woven bone, and fibrous tissue being present. Appositional new bone was observed on the surface (Figure 45) and within the macropores of TCP-HA particles. A pronounced fibrocellular reaction was present in many defects, consisting of a mixed population of macrophages, giant cells, plasma cells, and fibroblasts. Cells near TCP-HA granules were frequently packed with grains of TCP-HA.

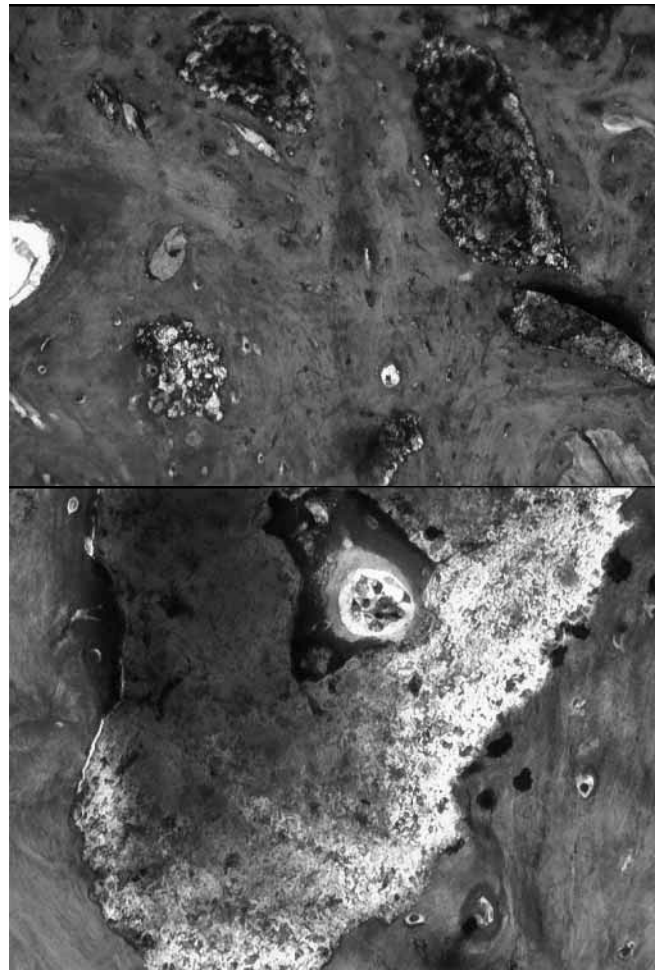


Figure 45: Undecalcified sections of metaphyseal defects *14 weeks after implantation of tricalcium phosphate-hydroxyapatite particles (toluidine blue and basic fuscin, X100).*
Top: particles surrounded by new bone
Bottom: revascularization and incorporation of particles

There was no evidence of bone cement penetration in any of the defects. Instead, the cement layer was separated from the defect by a fibrous tissue layer of variable thickness that was continuous with the periosteum.
A significant level of agreement ($p<0.001$) occurred between the qualitative grades assigned by the two observers, allowing the scores for graft incorporation to be combined for statistical analysis (Figure 46). The mean score of 1.7 (± 0.2) for defects implanted with "HA80" was significantly lower than the mean score of 2.6 (± 0.2) for defects treated with allograft alone. There was a trend for "TCP80" (BoneSave™) and "HA80-3sizes" to have higher scores than "HA80", but the difference did not reach significance.

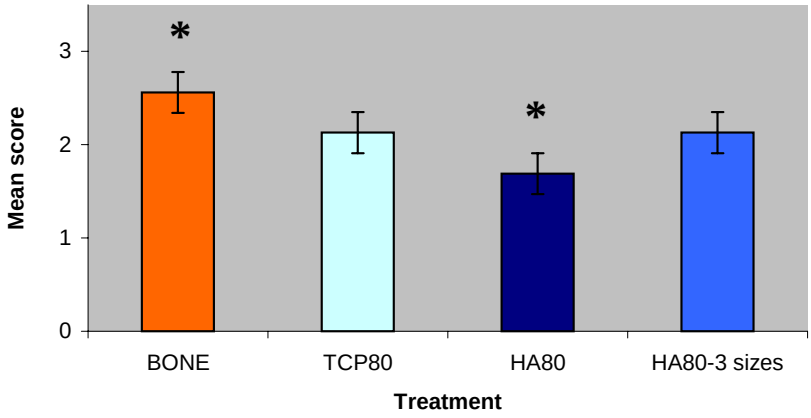


Figure 46: Histological grades *(mean) of metaphyseal defects filled with impacted morselized grafts. Grades increase from 1 to 3 with healing (mean ± Standard Error).*

- *Bone: impacted idealized morselized allograft*
- *TCP80: idealized mixture containing 80%TCP-20%HA (BoneSave™) in a 50/50 ratio with bone*
- *HA80: idealized mixture containing 80% HA-20%TCP in a 50/50 ratio with bone*
- *HA80-3sizes: partially idealized mixture containing 80%HA-20%TCP in a 50/50 ratio with bone*

** indicates a statistical difference between treatment groups*

8.5.3. Histomorphometry

Defects treated with allograft alone ("Bone") contained the highest proportion of bone elements, with a mean of 55.6 ± 4.1% of the defect area (Figure 47). The proportion of bone in defects treated with "HA80" was 38.0 ± 3.8%Ðsignificantly less than in defects treated with allograft

alone ($p<0.05$). There was a trend for defects treated with "TCP80" (BoneSave™) and "HA80-3 sizes" to have a higher proportion of bone than "TCP20-HA80", but the difference was not significant.

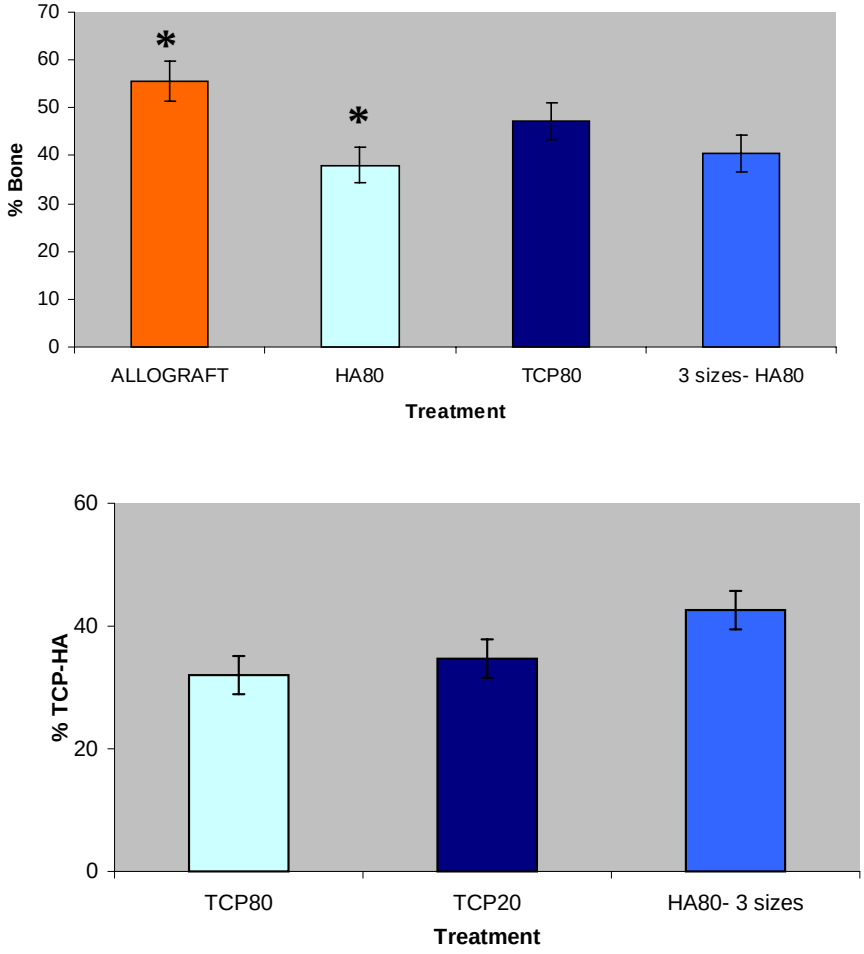


Figure 47: Histomorphometric analysis *- percentage of the defect area filled with bone and particles of synthetic agent (mean ± standard error).*

- *Bone: impacted idealized morselized allograft*
- *TCP80: idealized mixture containing 80%TCP-20%HA (BoneSave™), in a 50/50 ratio with bone*
- *HA80: idealized mixture containing 80% HA-20%TCP in a 50/50 ratio with bone*
- *HA80-3 sizes: partially idealized mixture containing 80%HA-20%TCP in a 50/50 ratio with bone*

* *indicates a statistical difference between treatment groups ($p<0.05$)*

No significant difference was present in the amount of TCP-HA remaining at 14 weeks amongst defects grafted with “TCP80”, “HA80”, and “HA80-3 sizes” (Figure 47).

8.5.4. Bone fluorescence

Mean mineral apposition rates for the early and late phase of graft incorporation are shown in Figures 48 and 49. No significant difference existed between treatments during early or late phases of defect healing ($p>0.05$).

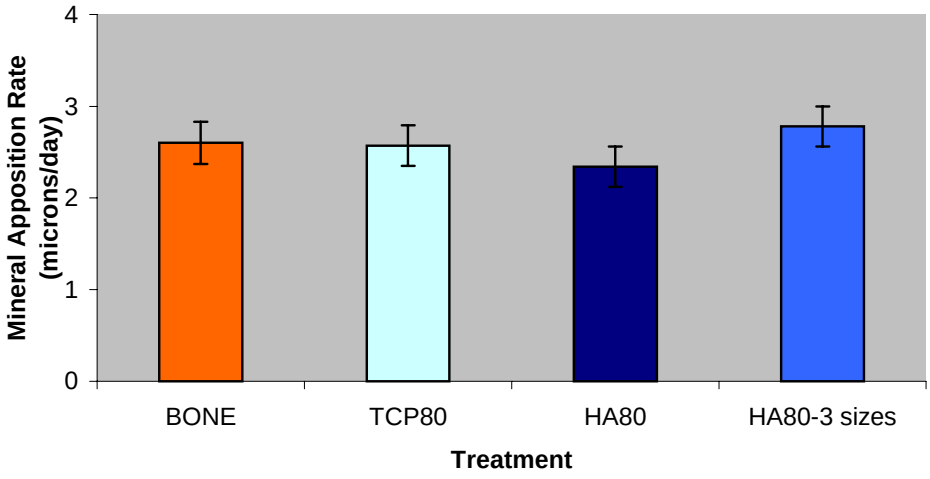


Figure 48: Mineral apposition rates measured by the double pulse administration of oxytetracycline, administered 23 and 35 days after surgery*, corresponding to the “early” phase of incorporation (mean± standard error).*

- *Bone: impacted idealized morselized allograft*
- *TCP80: idealized mixture containing 80%TCP-20%HA (BoneSave™) in a 50/50 ratio with bone*
- *HA80: idealized mixture containing 80% HA-20%TCP in a 50/50 ratio with bone*
- *HA80-3 sizes: partially idealized mixture containing 80%HA-20%TCP in a 50/50 ratio with bone*

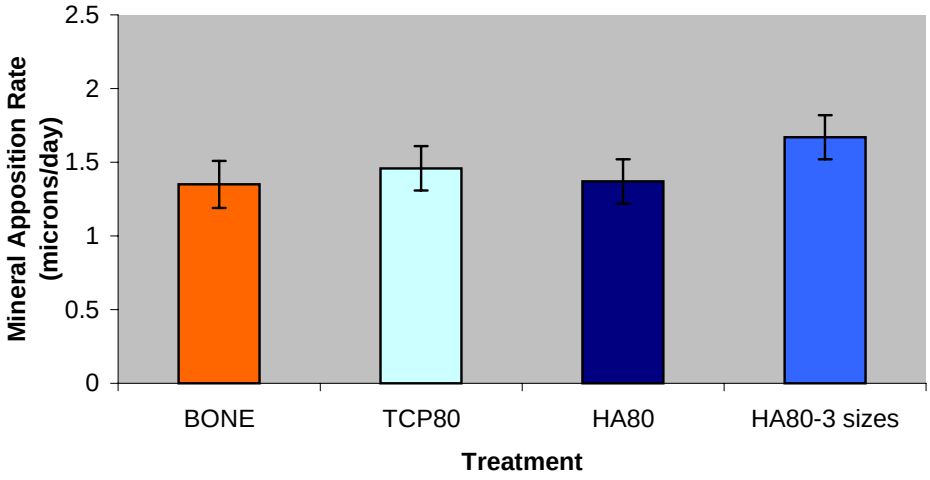


Figure 49: Mineral apposition rates measured between the administrations of oxytetracycline and alizarin, administered 35 and 84 days after surgery, respectively*, corresponding to the "late" phase of incorporation (mean ± standard error).*

9. DISCUSSION

9.1. BIOMECHANICAL PROPERTIES OF BONE FROZEN IN SALINE

Freezing of cortical allografts is a common method used for bone banking (Henry 1981, Denny 1985; Johnson 1988; Phillips et al. 1988; Ivory & Thomas 1993). Storage temperatures below -80°C are required to inhibit enzymatic processes responsible for cellular degradation (Bright & Burchardt 1983; Tomford 1983; Tomford et al. 1987). However, cortical specimens maintained at -20°C for several months have been used successfully in clinical cases (Henry 1981; Denny 1985; Sinibaldi 1989). Freezing at -20°C for up to one year does not appear to alter the biomechanical properties of cortical allografts, if surface hydration of tissues is preserved (Bright & Burchardt 1983; Roe et al. 1988; Tshamala et al. 1994). In our study, metacarpal and metatarsal bones frozen with or without NSS had similar biomechanical properties in torsion. In bending, the energy absorbed at failure by ribs and metacarpal and metatarsal bones stored in NSS was 25% to 30% greater than that of bones stored without NSS. This means that a larger amount of energy was necessary to fracture bones stored with sodium chloride and that they were tougher than bones frozen without it (Chao & Aro 1991). The bending displacement at failure of corticocancellous grafts frozen with NSS was 18% to 24% greater than that of grafts frozen without NSS. Ribs and metacarpal and metatarsal bones frozen without NSS are biomechanically more brittle. The reason the torsion properties of the grafts stored without NSS were preserved while their bending strength decreased remains unclear. Structural changes secondary to dehydration, such as different disposition of collagen fibers, may explain a selective decrease in the bending properties of these specimens.

The effects of dehydration on biomechanical properties of cortical bone are of special importance when evaluating freeze-drying as a method of bone banking. Freeze-dried bone is brittle and must be reconstituted with saline solution before use (Brown & Cruess 1982). After rehydration, compression strength is regained (Bright & Burchardt 1983) or slightly increased (Pelker et al. 1984) compared with that for fresh controls, but the torsion (Pelker et al. 1983) and bending strengths (Triantafyllou et al. 1975) remain substantially lower. Cortical grafts stored in dry packages are often rehydrated in saline solution (Brown & Cruess 1982; Denny 1985; Sinibaldi 1989; Johnson 1991; Stevenson 1991) immediately before transplantation, which may restore their biomechanical properties. In our study, bones were tested immediately after thawing and before rehydration to evaluate the effects of the storage technique alone.

Dehydrated bones have been reported to be brittle, hence, susceptible to splitting or flaking during placement of screws to immobilize the graft. Studies evaluating the effects of ethylene oxide sterilization and storage conditions on canine femora found a positive relation between available water and handling characteristics of bone undergoing drilling and tapping followed by seating of a cortical bone screw (Johnson et al. 1987). Use of polyethylene tubing was more efficient in preserving bone hydration than plastic film, paper, or plastic wrapping (Johnson et al. 1987). In our study, bones frozen in plastic packs without NSS were probably less tough and more brittle as a result of dehydration. Addition of NSS improved preservation of biomechanical properties of corticocancellous grafts. Freezing bone in NSS may be an alternative to use of polyethylene tubing as a packaging technique.

9.2. OSTEOPRODUCTIVE PROPERTIES OF A BONE-INDUCING AGENT IN DOGS

Isolation and purification of a bone-inducing agent from an established human cell line would provide an unlimited supply of osteoinductive bone graft substitute. Osteoinductive activity has been found in several animal (Takaoka et al. 1993) and human (Yan & Lianjia 1990; Htakeyama et al. 1993) neoplasms, but only a few of those have been established as cell lines (Fogh & Trempe 1975, Takaoka et al. 1989, Anderson et al. 1992). Live and freeze-dried neoplastic cells maintained in culture may induce bone formation after heterotopic implantation (Takaoka et al. 1989; Htakeyama et al. 1993). These preparations may also be carcinogenic (Htakeyama et al. 1993). This risk is reduced if the osteoinductive agent contained in the cells is extracted and purified. BIA has been extracted from Saos-2 human osteosarcoma cells. BIA contains a mixture of bone growth factors, most likely BMP-1, 2, 3, 4, 6, and Transforming Growth Factor-ß (TGF-ß) (Raval et al. 1993; Anderson et al. 1995).

In the study reported here, we confirmed the osteoinductive properties of BIA as it induced bone formation in muscle. Confirmation of osteoinductive properties of a bone-inducing agent requires ectopic implantation in a site void of osteoprogenitor cells. Muscle and subcutaneous tissue are the prevailing sites of implantation for osteoinductive bioassays. The latissimus dorsi muscle was selected for the osteoinductive bioassays because it is easy to expose and offers a large section of muscle of similar thickness. Bone formation is usually detected within two weeks after heterotopic implantation of an osteoinductive agent in athymic mice. In monkeys and dogs, osteoinductive bioassays are usually prolonged to six to eight weeks (Sato & Urist 1985; Miyamoto et al. 1993) to compensate for their slower metabolic rate compared to rodents (Coulson 1983; Sato & Urist 1985; Cook et al. 1994). The lack of bone formation after ectopic implantation of autogenous cancellous graft is in agreement with previous studies: nondecalcified bone transplants are minimally osteoinductive and generate the production of multinucleated cells (Chalmers et al. 1975; Nade 1979).

Although heterotopic implantation is necessary to determine the osteoinductive properties of a substance enhancing bone healing, the response to orthotopic implantation is more clinically pertinent (Kirker-Head 1995). Surgically created cortical bone defects have been used in dogs (Elkins & Jones 1988; Howard et al. 1988; Trevor et al. 1992; Forell & Straw 1993) to evaluate the effects of various treatments on bone healing. This model does not necessitate fixation and allows for evaluation of several local factors on the same bone. To limit variation of results due to location of the defects, all four defects were created in the diaphysis of a long bone and assigned to treatments according to a Latin square design.

The histological grades of the defects filled with autogenous cancellous bone are consistent with previous reports (Elkins & Jones 1988), with three of four defects being entirely filled with bone. Three of the four defects filled with a gelatin capsule alone had healed, whereas none of the defects treated with BIA had healed eight weeks after the operation. BIA stimulates early osseous union of a femoral nonunion in rats (Hunt et al. 1993), but 10 mg of BIA did not promote healing of cortical defects in dogs; no bone formation was found six weeks after intramuscular implantation of BIA. The failure of bone induction may be explained by faulty osteoinductive properties of the agent, an inadequate delivery of the agent, or the inability of dogs to respond to bone inductive stimuli that are effective in rodents.

For effective osteoinduction, osteogenic proteins must be implanted with an appropriate carrier substance since they are water soluble and readily diffusible. (Wozney 1993). The functions of the carrier include releasing the osteoinductive agent at an effective dose simultaneously with the accumulation of target cells, possibly protecting osteoinductive proteins against nonspecific proteolysis, and accommodating each step of the cellular response during bone formation. The carrier must also be biocompatible and biodegradable, and it should ideally provide a substrate for the attachment of recruited cells and blood vessel ingrowth (Cook et al. 1994). To evaluate the osteoinductive properties of an agent, the carrier must also be deprived of such properties. Pure bovine collagen type I did not induce ectopic bone formation in dogs. It is believed to act as a slow-release protein delivery system, improving the osteogenic potential of purified BMP *in vivo* (Bessho & Lizuka 1994). Bovine collagen has been used as a carrier for osteoinductive preparations in dogs (Cook et al. 1994) and failed to inhibit the osteoinductive properties of BIA in rodents. Therefore, the presence of bovine collagen type I is unlikely to account for the failure of BIA to induce bone formation in dogs.

The lack of osteoinductive effects of BIA after heterotopic and orthotopic implantation in the dog may be related to the dose of BIA used in this study. Induced bone formation is a dose-related phenomenon (Urist et al. 1987; Wozney et al. 1988; Gao et al. 1993). It has been suggested that in larger animals with longer lifespans, such as dogs, sheep, and monkeys, only bone and bone marrow contain perivascular mesenchymal cells able to respond to bone inductive signals (Nade & Burwell 1977; Sato & Urist 1985; Aspenberg et al. 1991). As a result, osteoinductive bioassays are generally performed in rodents and osteoinductive preparations are commonly evaluated in orthotopic sites in animals with a long lifespan (Sato & Urist 1985, Nilsson et al. 1986). Recent studies suggest that "long-lived" vertebrates retain extraskeletal inducible cells (Vail et al. 1994) and osteoinductive proteins in the extracellular matrix of bone (Forell & Straw 1993) but that the regenerative capacity of mammalian bone is inversely proportional to position on the phylogenetic scale (Aspenberg et al. 1992; Miyamoto et al. 1993; Cook et al. 1994). Higher vertebrates with a longer lifespan are less sensitive to osteogenic proteins than rodents. Nilsson et al. (1986) reported that 100 mg of partially purified bovine BMP, implanted in a gelatin capsule, induced radiographic healing of a canine ulnar defect at an average of 12 weeks. However, similar results were obtained with 625 µg of pure recombinant human morphogenetic protein-7 in the same nonunion model (Cook et al. 1994). The optimal dose of osteoinductive agent varies not only with the site and species of implantation but also with the nature of the agent (Sato & Urist 1985; Takaoka et al. 1993). BIA is sufficiently potent that doses as low as 1 mg induce ectopic bone formation in mice (Anderson et al. 1995), but dogs may require more than 10 mg of BIA to respond. Thus, evaluation of higher doses of BIA is indicated. The difference in results obtained after implantation of BIA in rodents versus dogs may also be related to immunogenic factors. The exact nature of the osteoinductive proteins in the extracellular bone matrix of different species is unknown, but the ability of demineralized bone matrix (DBM) to induce bone formation appears somewhat specific (Nilsson & Urist 1991; Forell et al. 1993). Athymic mice are suitable models for the osteoinductive bioassay of xenogeneic preparations because they are deprived of T-lymphocytes to initiate an immunogenic response to foreign material. Anti-BMP immunoglobulins were found in the serum of pigs two to three weeks after implantation of partially purified bovine bone morphogenetic protein fraction and associated noncollagenous proteins (Lindholm et al. 1994). An inflammatory response was initiated by implantation of demineralized bone matrix from various species in the rat, resulting in a lack

of osteoinduction (Sampath & Reddi 1983). Further purification of this xenogenic bone matrix and extraction of low molecular weight, noncollagenous proteins resulted in ectopic bone formation in the rat (Sampath & Reddi 1983). The bone morphogenetic protein fraction from diverse mammalian species is believed to consist of several homologous subunits (Bessho et al. 1992; Wozney 1993; Cook et al. 1994). The species specificity of bone induction is most likely attributable to immunogenic or inhibitory proteinaceous impurities (Sampath & Reddi 1983; Forell et al. 1993; Cook et al. 1994). BIA is a semi-purified agent, extracted from human cell lines, and further purification may be necessary to prevent an immunogenic response and allow osteoinduction in the dog.

9.3. EVALUATION OF AN OVINE MODEL TO TEST IMPACTED AGGREGATES

Unicortical bone defects are well-established models for evaluation of the biological properties of grafting materials. One of their limitations is related to the size of the defect and the risk of fracture secondary to a stress rising effect. Diaphyseal defects involving 20% of the diameter of the bone decrease the torsional strength by 34% (Edgerton et al. 1990). Whereas previous studies describe the use of diaphyseal unicortical defects measuring up to 9 mm in diameter in sheep (Frayssinet et al. 1993), the metaphysis of long bones can accommodate larger defects (Sartoris et al. 1986; Sauk & Van Kampen 1991). In our study, 15-mm-diameter metaphyseal defects were created to evaluate a representative sample of our aggregates, including particles up to 5 mm in diameter. The four femoral fractures observed in Study IV most likely resulted from the stress-rising effect created by the proximal and distal femoral defects. Limiting the number of defects to one per long bone and selecting larger sheep eliminated this complication in study V.

The other reason for selecting the metaphysis as the site of implantation was related to the balance between osteogenesis and biodegradation of grafting materials in cancellous bone. Lu et al. (1998) recently compared tissue reactions to calcium phosphate ceramics among cancellous, cortical, and medullar implantation sites in rabbits: medullar sites exhibited a stronger immune response and faster biomaterials degradation, whereas osteoproduction was increased within cortical sites. Cancellous sites had intermediate activities and were therefore recommended as models for evaluation of the properties of biomaterials.

Defects were covered with polymethylmethacrylate to simulate the environment of cemented revision hip arthroplasty, maintain the degree of impaction, and prevent migration of the pellets. Although grafting material was identified within all defects containing allograft, the cement seal migrated in 24.2% of defects in Study IV, while no migration was noted in Study V. The migration of cement seals in Study IV suggests that the amount of polymethylmethacrylate engaging empty screw holes was too small to maintain the plug in position. Embedding Kirshner wires was more effective than pouring liquid polymethylmethacrylate over empty screw holes in preventing migration of cement seals.

Although no difference was found between right and left limbs throughout the study, the site of implantation seemed to affect the incorporation of grafting material and healing of the defects. In Study IV, distal femoral defects contained more bone and less fibrous tissue than defects located in the proximal tibia seven weeks after surgery. Proximal femoral defects seemed to behave in way similar to distal femoral defects, but the depth of the intertrochanteric fossa in sheep is such that 15-mm-deep defects distal to the greater trochanter extended into the far cortex. Because this may affect the degree of compaction of pellets placed in that position, this site of implantation was eliminated in Study V. No difference was found between sites in terms of incorporation of grafting material, but the

synthetic agent evaluated in Study IV was completely resorbed in all defects but one, seven weeks after implantation. Similar findings were noted 14 weeks after surgery in Study V, where distal femoral defects obtained higher histological grades than tibial sites. Although this was not statistically significant, the amount of bone found within defects was greater in the distal femur than the proximal tibia. These results would suggest that defects heal faster in the former. However, the amount of synthetic agent was also higher in the distal femur than proximal tibia. These results correlate well with computed tomographic data, as particles of the agents tested in Study V were denser than allograft. The slower degradation of biomaterials and greater osteoproduction in distal femoral sites as compared with proximal tibia may be explained by a difference in tissue composition. Although this was not specifically measured, distal femoral sites appeared to contain more cancellous bone, whereas proximal tibial defects were closer to the medullar cavity. This would confirm previous studies in rabbits, where medullar sites exhibited faster degradation of biomaterials and less osteoproduction than cancellous sites (Lu et al. 1998). Our findings further suggest that the distal femur is a better site than the proximal femur and tibia to evaluate impacted morselized aggregates containing large particles in unicortical defects. However, this limits the number of treatment groups evaluated on the same animal. If different implantation sites are used within the same study, allocation of treatment to defects should follow a Latin square design to evaluate each treatment in all locations and eliminate variation due to position.

Mineral apposition rates measured within defects were overall 50% higher than remodeling rates 4 weeks after surgery, and decreased by 12 weeks in both phases of the study. In Study V, mineral apposition rates measured at 4 weeks were greater at the center than the periphery of the defect. This may reflect the progression of healing in unicortical bone defects, where bone formation proceeds from the periphery toward the center (Schenk & Hunziker 1994). Measurements obtained along the edges of the defect may therefore represent a more mature phase of healing, with lower mineral apposition rates than in the centre of the defect. To standardize our measurements, mineral apposition rates were obtained at the periphery of the superficial aspect for each implantation site in Study IV. In Study V, measurements obtained in four locations within each defect were averaged to compare treatments.

Although the model simulates some of the environmental characteristics encountered in impaction grafting for revision hip arthroplasty, it does not reproduce the mechanical loading generated in clinical cases. Biomechanical loading has recently been shown to affect the incorporation of morselized bone graft in a unicortical bone defect (Lamerigts et al. 2000). In this model, controlled compression was applied over the defect area, whereas grafting material impacted around a femoral stem is exposed to a combination of compression and shear forces.

9.4. BIOLOGICAL PROPERTIES OF A SILICATE-FREE BIOACTIVE GLASS

Although site of implantation has been found to influence the osteoproduction and degradation rate of implants (Lu et al. 1998 and section 9.3.), randomization of treatment allocation according to a Latin square design allows us to compare treatment groups. Our results suggest that healing of metaphyseal defects tended to decrease with increasing concentrations of Corglaes®. Defects filled with 50/50 or 100% Corglaes® contained more fibrous tissue and less bone than defects filled with bone 14 weeks after implantation. Removal of Corglaes® particles consists of chemical dissolution, resulting in an acidic environment (Gilchrist et al. 1991). It is possible that rapid dissolution of high concentrations of Corglaes® (50% or 100%) overwhelmed the physiologic buffering systems and resulted in a

decrease of local pH, stimulating osteoclastic activity and affecting healing of the defects (Hruska et al. 1993). Allograft also compared favorably to synthetic agents in other studies, justifying its use as a control (Moore et al. 1987; Virolainen et al. 1997). However, limitations associated with the use of allografts may eventually outweigh the benefit of higher osteoproductive properties. Interestingly, none of the parameters evaluated in our study differed between defects filled with allograft and those filled with allograft idealized with Corglaes®. This mixture, containing approximately 25% Corglaes®, would have the biomechanical advantage of improved resistance to shear compared with allograft, and therefore warrants further evaluation.

Our results also suggest that morselized Corglaes® was nearly dissolved by seven weeks after impaction in sheep metaphyseal defects. Indeed, remnants of bioactive glass were found in only one defect, seven weeks after implantation. There was no evidence of synthetic agent at 14 weeks. Incorrect sectioning or migration of pellets seem unlikely since particles of dead bone were identified in all defects containing allograft alone, or mixed with the synthetic agent. This early dissolution would explain the computed tomography findings. Although pellets containing Corglaes® had higher pixel values than allograft pellets prior to implantation, defects treated with 50% or more Corglaes® were less dense than defects filled with allograft, seven weeks after surgery. The incorporation of Corglaes® in our study seems faster than that of previously evaluated bioactive glasses (Gao et al. 1995; Schepers & Ducheyne 1997; Hench 1998). However, properties of bioactive glasses are influenced by their composition, manufacturing process, and presentation. The main difference between the controlled release glass (CRG) evaluated in our study and previous bioactive glasses is that it contains biodegradable P_2O_5 rather than SiO_2 as a network former. A typical CRG will comprise 42-49 mole% of P_2O_5, the remainder as 10-40 mole% of CaO and Na_2O. Variation in ratios and addition of various compounds can be used to adjust the dissolution rate. For example, increased levels of CaO, decreased concentrations of Na_2O, and fluoride additions will slow the dissolution of the compound (Burnie & Gilchrist 1983; Hench 1998). Annealing temperature is one aspect of the manufacturing process that will affect the longevity of biodegradable implants, with higher temperatures slowing the degradation of calcium phosphate fibers (Choueka et al. 1995). Granulometry has also been shown to influence the biological properties of bioactive glass (Schepers & Ducheyne 1997) as well as other ceramics (Malard et al. 1999): smaller particles were associated with faster degradation and higher osteoconductive properties. Therefore, an aggregate combining particles of different sizes could potentially benefit from prolonged osteoconduction associated with a gradual degradation process. The other advantage consists of improved biomechanical properties associated with increased cohesion between particles, when their size distribution follows the ideal linear logarithmic grading line (Brewster et al. 1999). The ideal integration rate of grafts used in revision hip arthroplasty has not been determined but should probably be longer than seven weeks to allow gradual replacement by new bone and remodeling. Degradation of grafting material may however be slower along the smooth, poorly vascularised endosteal surface of a revised femoral diaphysis compared with the normal metaphyseal cancellous bone in our study. Differences in osmotic fluxes, vascularity, and biomechanical loads between our model and the clinical setting may also influence the incorporation of synthetic agents (Tagil 2000).

9.5. OSTEOCONDUCTIVE PROPERTIES OF IMPACTED MORSELIZED TRICALCIUM PHOSPHATE-HYDROXYAPATITE

A metaphyseal defect model was selected for this study to allow preliminary evaluation of the biological properties of several agents within the same animal. This model is technically simple to use and, while simulating some of the environmental characteristics encountered in revision hip arthroplasty (contact with cancellous bone and cement, impaction of grafting materials), is not intended to mimic the loading conditions encountered clinically. Although site of implantation has been found to influence the osteoproduction and degradation rate of implants (Lu et al. 1998 and section 9.3.), randomization of treatment allocation allows us to compare treatment groups.

Corticocancellous allograft was chosen as one treatment since it is currently used in human impaction grafting. Idealized, impacted corticocancellous allograft was well incorporated within metaphyseal defects: by 14 weeks, the original trabecular architecture had been restored and the majority of dead bone had been removed or remodeled. The larger remaining fragments tended to be remnants of morselized cortical bone. The presence of cortical bone is not necessarily undesirable since it may contribute to the stability of the aggregate as the allograft becomes incorporated. Our results compare favorably with other experimental studies using impacted allograft (Tagil & Aspenberg 1998; Lamerigts et al. 2000; Tagil 2000).

The mean CT-derived density of defects treated with allograft alone was significantly lower than that of defects treated with mixtures containing synthetic agents. The higher density of TCP-HA granules as compared with morselized allograft can explain these results. No significant difference in density was seen between the three TCP-HA expanded mixtures. Similarly, there was no significant difference in the mean percentage of residual synthetic implants within defects. These two results suggest that the dissolution rate for 80%TCP-20%HA is similar to 80%HA-20%TCP at 14 weeks. We expected 80%TCP-20%HA to be removed more rapidly since TCP is more soluble than HA in tissue fluids. However, both of these materials are ceramics, which, like HA are relatively inert and mostly removed by cellular processes (Lamerigts et al. 2000). On histological examination, little difference was found in the degree of cellular degradation between the different ceramic mixtures, and particles were seen within phagocytic cells in all defects treated with TCP-HA mixtures.

Our results confirm that corticocancellous allograft expanded with TCP-HA particles was osteoconductive. However, the degree of incorporation within and between TCP-HA treatment groups was variable and less consistent than for allograft alone. The lowest histological grades and proportion of bone within the defect were obtained with the fully idealized mixture of particles containing 80% HA. On the other hand, no difference was found between sites implanted with a fully idealized mixture of particles containing 80% TCP, the partially idealized mixture (3 sizes) containing 80% HA, and the positive control (allograft).

There was a trend for particles containing 80% TCP to have a higher mean incorporation grade and more bone within defects than particles rich in HA. Both of these mixtures were idealized, but differed in the relative proportions of TCP and HA. A higher proportion of the more soluble TCP appears to improve osteoconduction. Our results conflict with the results of a study evaluating biphasic calcium phosphate granules for repair of periodontal osseous defects, where 15%TCP-85%HA granules gave superior results to all other ratios of TCP to HA that were evaluated (Frayssinet et al. 1993).

In the present study, we also applied the concept of “idealization” to impacted test mixtures. Compaction of conventional allograft may cause a transitory reduction in osteoconduction

(Tagil et al. 1998; Tagil 2000). Idealization increases cohesion between particles and may therefore impair incorporation of the graft as well as its osteoconductive properties. Defects filled with a mixture idealized with three particle sizes (partially idealized) tended to reach a higher mean histological grade and contained more bone than sites implanted with a mixture idealized with eight particle sizes. The chemical composition of the TCP-HA granules was identical and the two mixtures differed only by the degree of idealization. This suggests that a less stringent idealization process may enhance osteoconduction, possibly because the structure of the mixture is more "open". A mixture prepared with particles of three sizes instead of eight is also easier to manufacture. A partially idealized mixture of particles containing 80% TCP studies warrants further evaluation in a hemiarthroplasty model, simulating the loading conditions encountered clinically (Blom et al. 2001).

10. CONCLUSIONS, CLINICAL SIGNIFICANCE, AND FUTURE DIRECTIONS

Study I:

In the first study, bones frozen in plastic packs without normal saline were probably less tough and more brittle as a result of dehydration. Addition of isotonic saline solution improved preservation of biomechanical properties of corticocancellous grafts. Freezing bone in normal saline solution may be an alternative to use of polyethylene tubing as a packaging technique.

Clinical significance:
Results of this study suggest potential benefits in long-term freezing of corticocancellous bone in isotonic saline solution. Ribs and metacarpal and metatarsal bones stored in normal saline solution were comparatively tougher and less brittle than grafts stored without isotonic saline, when tested to failure in bending. However, biomechanical testing of cortical bone differs from the clinical situation because it does not address ongoing biological processes associated with revascularization, incorporation, and remodeling of the graft.

Future directions:
Secondary sterilization of bone allograft by gamma irradiation affects the biomechanical properties of bone allograft. Bone irradiated at 30 kGy, which is the dose recommended to inactivate HIV (Fideler et al. 1994), becomes so brittle that it cannot be used as a structural graft (Pelker & Friedlander 1987). These changes result from the dose-dependent effect of irradiation on the plastic properties of collagen. Radiation produces reactive free radicals by radiolysis of water, which damages collagen alpha chains (Stachowicz et al. 1970). The effects of irradiation and the degree of inactivation of HIV in culture may vary with factors such as temperature. In a recent study, bone irradiated at -78°C (i.e. frozen) was less brittle and sustained less collagen that when irradiated at room temperature (Hamer et al. 1999). It would be interesting to study whether immersion in saline solution would improve the effects of irradiation and decrease the dose required for HIV inactivation, thus preserving the biomechanical properties of allogenic bone.

Study II:

Ten milligrams of the bone-inducing agent extracted from a human osteosarcoma cell-line (Saos-2) was not osteoinductive and did not promote bone healing after orthotopic implantation in dogs. The difference between these results and those obtained in rodents may be related to an immune reaction or an insufficient dose of BIA. The human origin of BIA may have been the source of immunogenical factors, inhibiting osteoinduction in dogs.

Clinical significance:
Although the human origin of BIA may be an advantage for use in man, further evaluation in immunocompetent higher vertebrates is warranted prior to human clinical trials. Such tests could involve higher doses of the agent or evaluation in nonhuman primates.
Further to this study, tests confirmed the presence of immunogenic impurities in the preparation.

Future directions:
The culture medium of Saos-2 cells appears osteoinductive in rodents (Anderson HC, personal communication). This may represent an even more effective and potentially safer (the medium does not contain neoplastic cells) method to produce a mixture of growth factors and warrants further investigation.
This project has generally raised my interest in osteoinductive factors present in bone tumors. Whereas significant research has focused on the relationship between bone morphogenetic proteins and neoplastic bone diseases in man (Bauer & Urist 1981; Yan & Lianjia 1990; Yoshikawa 1994 a,b), very little has been done in veterinary medicine. A comparative study of the expression of BMPs in canine and feline osteosarcoma would open up an unlimited field of research. For example, it would be interesting to determine whether the presence of BMP activity in small animal osteosarcoma has the same prognostic value as in man (Yoshikawa et al. 1985, 1988). This type of study could have consequences on the clinical management of bone tumors.

Study III:

We describe a model for preliminary evaluation of the biological properties of impacted morselized grafts previously studied *in vitro*, and considered for use in revision hip arthroplasty. This model does not require internal fixation, optimizes the use of animals, and accommodates aggregates consisting of large particles. Whereas no difference was found between left and right limbs, osteogenesis and incorporation of biomaterials varied between implantation sites. Treatment site allocation should therefore be randomized according to a Latin square design for comparison of grafting materials. The distal femur provided the best location in our study as it subjectively contained more cancellous bone than the proximal tibia and provided an intact far cortex compared with the proximal femur. Only one defect (15 mm in diameter and depth) should be created in each long bone (femur and tibia) to prevent postoperative fractures. Idealized morselized impacted allograft provides a positive control for comparison of treatment groups using computed tomography, histology, and histomorphometry. The location for measuring mineral apposition rates should be standardized within each defect.

Clinical significance:
This model is particularly useful for selecting grafting materials prior to evaluation in a hemiarthroplasty model, simulating the loading conditions encountered clinically.
A mixture of Corglaes® and allograft is currently being tested in an ovine hemiarthroplasty model (Dunlop & Field 2000). On histological evaluation of a few specimen, particles of bioactive glass are seen near the distal end of the stem (femoral diaphysis), whereas no synthetic agent can be found in the proximal femoral canal at 14 weeks after implantation (Figure 50). This preliminary data suggests that the behaviour of grafting material may be similar in the ovine metaphyseal defect model and the proximal femur after impaction grafting hip arthroplasty.

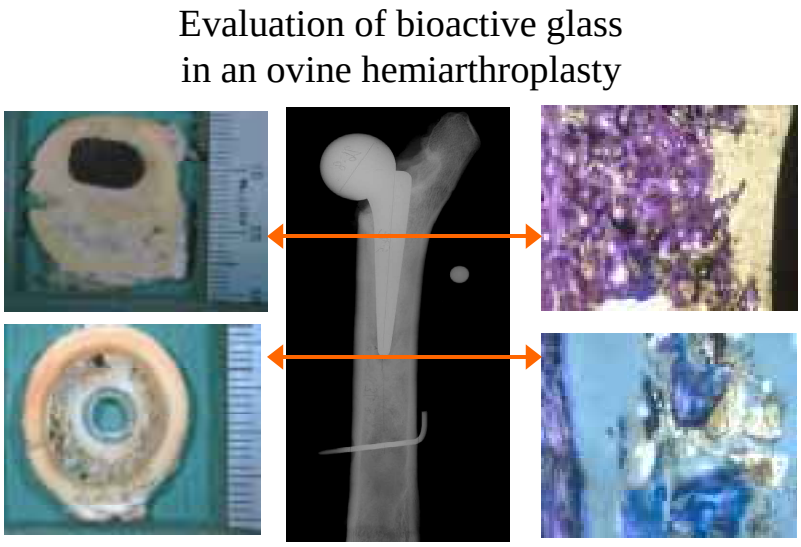


Figure 50: Radiograph, gross appearance, and histology of an ovine femur 14 weeks after impaction grafting hemiarthroplasty. *A collarless polished tapered femoral stem (with centralizer) was implanted after impaction of a 50/50 allograft/bioactive glass idealized mixture in the femoral canal. Particles of bioactive glass are only found near the distal end of the implant. (Courtesy of D Dunlop).*

Study IV:

Corglaes® implanted in ovine metaphyseal defects was resorbed faster than predicted i*n vitro*, within 7 weeks, which is too fast for use in impaction grafting arthroplasty. Healing of defects containing 50% or 100% synthetic agent was poorer than defects treated with allograft. On the other hand, no difference was found between sites implanted with bone (autograft or allograft) and allograft idealised with Corglaes®.

Clinical significance:
Further evaluation of Corglaes® is warranted before clinical trials may be considered.
Based on our results, evaluation of an idealized mixture of bone and Corglaes® in a hemiarthroplasty model, which would reproduce the biological and biomechanical conditions encountered clinically seems warranted. If our results are confirmed, the composition and/or manufacturing process of the compound tested in our study should be revised before it can be considered for impaction grafting in revision hip arthroplasty.

Future directions:
The dissolution rate of the agent tested is compatible with the delivery of local growth factors and antibiotics. It may therefore be considered as a potential carrier for local delivery of antibiotics in skeletal tissues, as described for traditional bioactive glasses (Otsuka et al. 1995), and is already used in other medical devices as a broad-spectrum antimicrobial metal ion delivery system, shown in Figure 51 (Gilchrist et al. 1991).

Figure 51: Buttons of Corglaes® loaded with antimicrobial agents such as zinc, copper, or silver.

Based on the results of this study, the manufacturer has modified the attenuation factor used to predict *in vivo* dissolution rate based on *in vitro* tests. The composition of the agent is also modified to produce a glass that should dissolve in about six months after implantation. *in vivo* evaluation of the new agent will be indicated to verify its biological properties. There is some evidence to suggest that particles of traditional bioactive glass measuring less than 300 μm may be more osteoconductive than larger particles (Schepers et al. 1991; Nasr et al. 1999). It would therefore be interesting to evaluate whether the addition of smaller particles (less than 300 μm) of silicate-free glass to an idealized mixture would influence its properties. Another pole of research interest focuses on the production of osteoconductive fibers. Although it has not been confirmed, this presentation may increase osteoconductive properties of bone graft substitutes and modify their biomechanical properties. None of the polymer-coated bioactive glass (silicate-free) fibers produced has been evaluated for orthopedic use.

Study V:

Implantation of idealized, impacted, morselized allograft resulted in healing of defects at 14 weeks. Impacted, idealized mixtures have been shown elsewhere to have superior biomechanical properties when compared with conventionally impacted allograft (Brewster et al. 1999). Expanding allograft with tricalcium phosphate - hydroxyapatite granules to produce an idealised mixture offers many advantages and shows promise. Partially idealizing the mixture may improve graft incorporation, with implications for easier manufacture. Particles containing 80% tricalcium phosphate tended to be more osteoconductive than particles containing 80% hydroxyapatite. Additional studies are in progress to determine the long-term behavior of these mixtures under realistic loading conditions in a sheep arthroplasty model (Blom et al. 2001).

Clinical significance:
Particles containing 80%TCP-20%HA have been approved for use in Europe. The agent is currently being launched as an allograft expander for impaction grafting hip arthroplasty under the name of BoneSave®. It is available in two size ranges: 2- to 4- mm-diameter particles are recommended for use in femoral impaction grafting, whereas 4- to 6- mm-diameter particles are recommended for use in acetabular impaction grafting. BoneSave® is now undergoing clinical trials in selected centers throughout Europe.

Future directions:
The agent may be considered for use in impaction grafting knee replacement. Another direction of research may focus on its use as a carrier for local growth factors. Although, collagen has been the most common delivery system for BMPs, its biomechanical properties preclude its use as a graft expander in impaction grafting (Lindholm & Gao 1993; Wolfe & Cook 1994; Ladd & Pliam 1999). On the other hand, synthetic ceramics have been used as carriers for BMPs (Herr et al. 1993; Lindholm & Gao 1993; Ripamonti 1993; Hotz & Herr 1994; Laffargue et al. 1999; Tay et al. 1999). It would therefore be interesting to evaluate the applicability of TCP-HA augmented with rhBMP-2 in impaction grafting. Because the implants used in impaction grafting hip arthroplasty are most frequently cemented, a preliminary study should be aimed at measuring the minimum distance from heat-generating bone cement required to preserve the osteoinductive properties of a graft.

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